August 1, 2003

Dockets Management Branch, Food and Drug Administration, Department of Health and Human Services, Room 1-23, 12420 Parklawn Dr., Rockville, MD 20857 CITIZEN PETITION. The undersigned submits this petition under 21CFR Part 10.30 of the Federal Food, Drug, and Cosmetic Act, the Public Health Service Act, or any other statutory provision for which authority has been delegated to the Commissioner of Food and Drugs (under 21 CFR, 5.10) to request the Commissioner of Food and Drugs to amend a regulation.

A. ACTION REQUESTED

Request 1.

Update the Drug/Laboratory Test Interactions section of the prescribing information of all strengths of Premarin tablets (.3mg, .45mg, .625mg, 1.25mg, 2.5mg) to recognize the decreased levels of testosterone with oral estrogen use. The following wording would be appropriate:

Replace:

"Free or biologically active hormone concentrations are unchanged.

With:

"Free or biologically active hormone levels, i.e., testosterone, are reduced"

Request 2.

List all known active components of Premarin in the description section of the prescribing information.

Replace:

"The conjugated equine estrogens found in Premarin tablets are a mixture of sodium estrone sulfate and sodium equilin sulfate. They contain as concomitant components, as sodium sulfate conjugates, 17 alpha-dihydroequilin, 17 alpha-estradiol, 17 beta-dihydroequilin"

With:

"The conjugated equine estrogens, androgens and progesterones found in Premarin are a mixture of estrone, 17 beta-estradiol, 17 alpha-estradiol, equilin, equilenin, 17 alpha-dihydroequilin, 17 beta-dihydroequilin, 17alpha-

2003P-0357

CP1

dihydroequilenin, 17 beta-dihydroequilenin, delta-8-estrone, estradiene, 6 identified but unnamed estrogenic compounds, 4 identified but unnamed androgenic compounds, and 5 identified but unnamed progestogenic compounds"

B. STATEMENT OF GROUNDS

Grounds for Request 1.

Recently, while researching hormone replacement products, I found discrepancies in the prescribing information of Premarin. This is important because the inaccurate information may lead to deleterious outcomes.

It is accepted that oral estrogens taken either for birth control or hormone replacement therapy significantly increase sex hormone binding globulin, SHBG (200%-300%). Even very low dosages (.3mg) can induce this binding protein significantly. It is also accepted that estrogen given transdermally has a minimal effect on increasing SHBG levels (3-10%). [1,2.3]

There is a discrepancy in the description of how SHBG and free testosterone interact. The current information states that there may be an increase in SHBG, but that the bio-availability of testosterone, i.e. the free testosterone level, does not change. As the ratio of free to total testosterone is directly related to SHBG levels, this statement implies that there is a rise in total testosterone corresponding with the rise in SHBG. However, it has been shown that total testosterone does not rise when taking estrogens, either orally or transdermally.[4] If the SHBG goes up, but the total testosterone remains constant, the free or bio-available testosterone levels must decrease. The graph below (Fig. 1.) from "*Associations among oral estrogen, free testosterone concentration, and lean body mass among postmenopausal women*" (Gower and Nyman, 2000) illustrates how the total testosterone remains level with oral estrogen use however the free testosterone level is reduced while SHBG is increased.

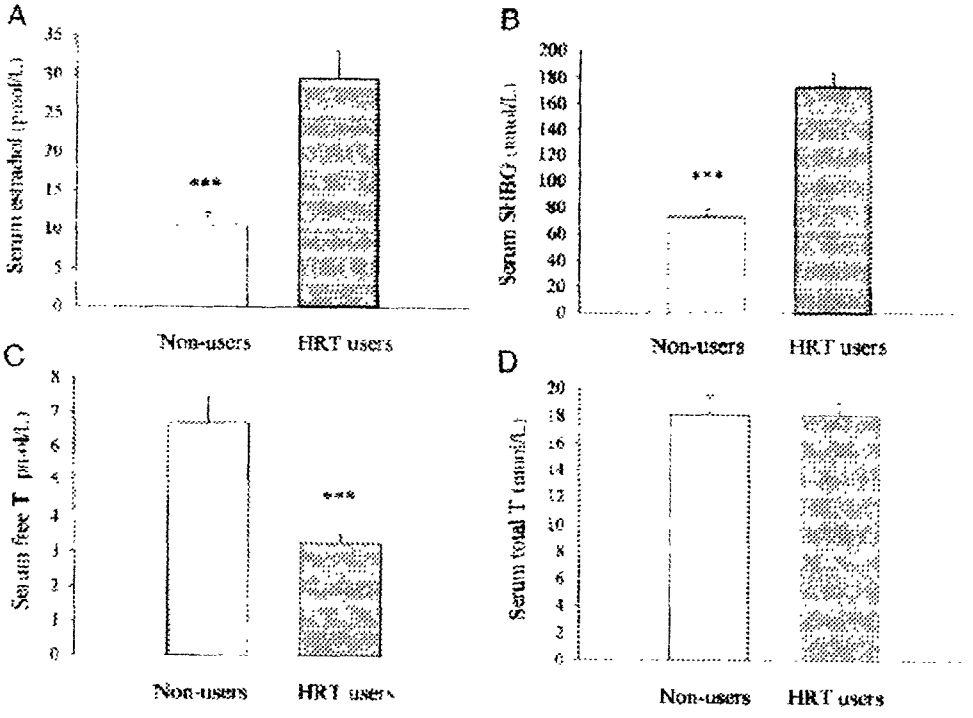

Figure 1A. Serum E_2 concentration (pmol/L) in users and nonusers of HRT;*** P<0.01. B. Serum SHBG concentration (nmol/L) in users and nonusers of HRT; *** P<0.01 C Serum free T concentration (pmol/L) in users and nonusers of HRT; *** P < 0 01 D. Serum total T concentration (nmol/L) in users and nonusers of HRT: means were not significantly different

In "*Transdermal Testosterone for Women: A New Physiological Approach for Androgen Therapy*" (2003), Mazer and Shifren discuss the effects of a testosterone patch as used by both pre and post menopausal women. They examine these effects without simultaneous estrogen therapy and in the presence of both oral and transdermal estrogen therapies. They conclude that "by markedly raising SHBG levels, the concomitant use of oral estrogen therapy increases the total and decreases the free concentrations of testosterone achieved with the testosterone matrix patch. Transdermal estrogen therapy does not alter SHBG concentrations and does not seem to influence testosterone levels in this way."

The prescribing information for Premarin states that there may be an increase in the serum level of SHBG. This is incorrect as many studies have shown that oral estrogen significantly increases SHBG while transdermal has a minimal effect on SHBG.

A problem may arise for patients using estrogen and testosterone supplementation together. When switching from an oral estrogen preparation to a transdermal one, the patient's SHBG would drop significantly. Because the SHBG would no longer be artificially raised, continuing testosterone supplementation at the same dosage could cause the level of serum bio-available testosterone to rise to unacceptable levels.

Androgen therapy is commonly prescribed along with estrogen replacement therapy. Therefore, accurate, consistent information for the prescribing physician must be available to prevent an adverse drug interaction.

Therefore, in accordance with 21 CFR 201.56 subsection (b), this side effect of Premarin should be listed in the label.

Grounds for Request 2.

The Chromatogram below (Fig 2.), from "*Recent insights into the varying activity of estrogens*," (Dey, Lyttle and Pickar, 2000) identifies 17 known estrogenic components, 4 known androgenic components, and 5 known progestogenic components of Premarin. Each peak has been identified as either estrogenic, androgenic, or progestogenic and appropriately labeled. As such the components labeled in the chromatogram would stimulate estrogen, androgen or progesterone receptors respectively, thereby making them active components of Premarin. All active components of Premarin should be listed in the description section of the prescribing information.

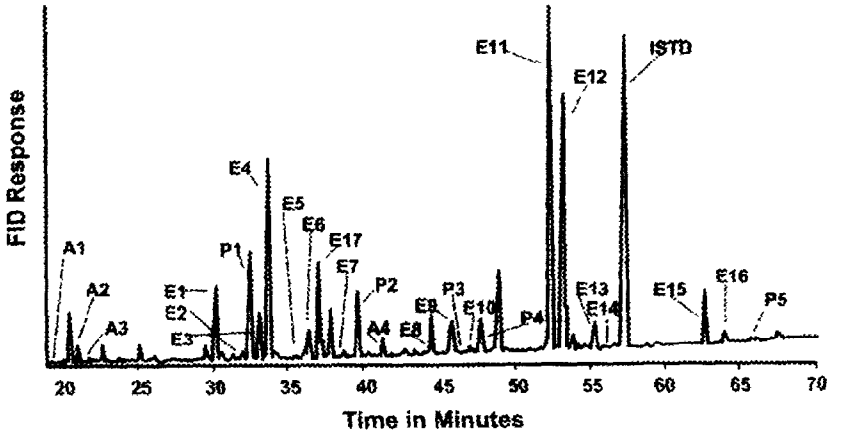

Figure 2. Chromatogram that identifies the complex mixture of estrogenic (E), progestogenic (P) and androgenic (A) components of Premarin

In the paper, the authors refer to Figure 2 which was produced using high pressure liquid chromatography/mass spectrometry. This graph "revealed not only estrogens but also androgens and progestins in Premarin." Additionally, this paper introduces a new estrogen, estradiene, "the fourth most abundant estrogen in Premarin. It is present in amounts three times greater than that of delta (8,9)-dehydroestrone." The authors go on to describe the potency of estradiene as very similar to that of delta (8,9)-dehydroestrone, a potent uterotropic component of Premarin. Wyeth-Ayerst Laboratories should be familiar with this data as the afore mentioned study was done by Wyeth-Ayerst Research.

The components of Premarin are well studied and its components are published in many medical journals.

Here is a partial list of papers which list the ingredients of Premarin products:

1) "*Recent insights into the varying activity of estrogens*", 2000, Maturitas. Apr 1;34 Suppl 2:S25-33.

1) 17 beta-estradiol
2) 17 beta-Dihydroequilin
3) 17 beta-dihydroequilenin
4) 17 alpha-dihydroequilin
5) 17 alpha-estradiol
6) Estrone
7) Equilin
8) 17 alpha-dihydroequilenin
9) Delta8,9-dehydroestrone (DHES)
10) Equilenin
11) Estradiene

2) "*Pharmacokinetics and Pharmacodynamics of a Novel Estrogen Delta-8-Estrone in Postmenopausal Women and Men*", 1998, J Steroid Biochem Mol Biol. Oct;67(2):119-31.

1) Equilin
2) Equilenin
3) Estrone
4) 17 beta-dihydroequilin
5) 17 beta-dihydroequilenin
6) 17 beta-estradiol
7) Delta-8 estrone
8) 17 alpha -dihydroequilin
9) 17 alpha -dihydroequilenin
10) 17 alpha-estradiol

This paper also includes the molecular structure of these compounds.

3) "*Pharmacokinetics and Pharmacodynamics of Conjugated Equine Estrogens: Chemistry and Metabolism*", 1998, Proc Soc Exp Biol Med. Jan;217(1):6-16

1) Equilin
2) Equilenin
3) Estrone
4) Delta-8-estrone
5) 17 beta-dihydroequilin
6) 17 beta-dihydroequilenin
7) 17 beta-estradiol
8) 17 alpha-dihydroequilin
9) 17 alpha-dihydroequilenin
10) alpha-estradiol

4) "*Biologic Effects of Delta-8-Estrone Sulfate in Postmenopausal Women*", 1998, J Soc Gynecol Investig. May-Jun;5(3):156-60.

1) Estrone
2) 17 beta-estradiol
3) 17 alpha-estradiol
4) 17 alpha-estradiol
5) Equilenin
6) 17 alpha-dihydroequilin
7) 17 beta-Dihydroequilin
8) 17 alpha-dihydroequilenin
9) 17 alpha-dihydroequilenin
10) 17 beta-dihydroequilenin

In this paper the following is list of ingredients of Premarin includes "the presence of a tenth ring B unsaturated estrogen component identified as delta-8-estrone now has been confirmed in Premarin. Delta-8-E_1 is the fifth most abundant estrogen component in Premarin and makes up approximately 3.5% of the total estrogen present in a Premarin tablet".

5) "*Pharmacokinetics of Equilin and Equilin Sulfate in Normal Postmenopausal Women and Men*", 1983, J Clin Endocrinol Metab. May;56(5):1048-56.

1) Estrone
2) 17 alpha-dihydroequilin
3) 17 alpha-dihydroequilenin
4) 17 alpha-estradiol
5) 17 beta-estradiol
6) 17 beta-dihydroequilin
7) 17 beta-dihydroequilenin

Clearly more components are identified than are listed in the prescribing information for Premarin.

In their 1994 citizen petition, Wyeth-Ayerst requested that DHES be listed as an essential ingredient of Premarin. They later amended their petition to have DHES be listed as an essential active ingredient. To date, this petition has not been acted upon. In 1996, at the request of the Chairman, Subcommittee on Oversight and Investigations, House Committee on Commerce, the Office of the Inspector General (OIG) of the Department of Health and Human Services investigated the handling of this petition. In the summary report, "*Review of the Food and Drug Administration's Handling of Issues Related to Conjugated Estrogens (A-15-96-50002)*", the OIG concluded that DHES was in fact an essential and active ingredient of Premarin.

Therefore, in accordance with 21 CFR 201.100 subsection (b)(2), these 21 other components (12 estrogenic, 4 androgenic, 5 progestogenic) must be included on the labeling along with their relative percentages.

C. ENVIRONMENTAL IMPACT STATEMENT

No environmental impact statement is required for this petition under 21 CFR 25.30 subsection k. The proposal is for a labeling change that does not change the level of use or the intended use of the product.

D. ECONOMIC IMPACT STATEMENT

No economic impact statement has been requested.

E. CERTIFICATION

The undersigned certifies that, to the best knowledge and belief of the undersigned, this petition includes all information and views on which the petition relies, and that it includes representative data and information known to the petitioner which are unfavorable to the petition.

Signature

Beth Rosenshein
15149 SE 48th Dr, Bellevue, WA 98006
(425) 603-0793

Encl.

1. Fahraeus L, Larsson-Cohn U., 1982, *Oestrogens, gonadotrophins and SHBG during oral and cutaneous administration of oestradiol-17 beta to menopausal women.*, Acta Endocrinol (Copenh). Dec;101(4):592-6.

2. Serin IS, Ozcelik B, Basbug M, Aygen E, Kula M, Erez R., 2001, *Long-term effects of continuous oral and transdermal estrogen replacement therapy on sex hormone binding globulin and free testosterone levels.*, Eur J Obstet Gynecol Reprod Biol Dec 1;99(2):222-5.

3. Slater CC, Zhang C, Hodis HN, Mack WJ, Boostanfar R, Shoupe D, Paulson RJ, Stanczyk FZ., 2001, *Comparison of estrogen and androgen levels after oral estrogen replacement therapy.*, J Reprod Med. Dec;46(12):1052-6.

4. Gower, BA, Nyman L., 2000, *Associations among oral estrogen use, free testosterone concentration, and lean body mass among postmenopausal women.*, J Clin Endocrinol Metab. Dec;85(12):4476-80.

5. Mazer NA, Shifren JL. 2003, *Transdermal testosterone for women: a new physiological approach for androgen therapy.*, Obstet Gynecol Surv. 2003 Jul;58(7):489-500.

6. Dey M, Lyttle RC, Pickar JH., 2000, *Recent insights into the varying activity of estrogens.*, Maturitas. 2000 Apr 1;34 Suppl 2:S25-33.

7. Bhavnani BR, Cecutti A, Gerulath A., 1998, *Pharmacokinetics and Pharmacodynamics of a Novel Estrogen Delta-8-Estrone in Postmenopausal Women and Men.*, J Steroid Biochem Mol Biol. Oct;67(2):119-31.

8. Bhavnani BR., 1998, *Pharmacokinetics and Pharmacodynamics of Conjugated Equine Estrogens: Chemistry and Metabolism.*, Proc Soc Exp Biol Med. Jan;217(1):6-16.

9. Bhavnani BR, Cecutti A, Dey MS., 1998, *Biologic Effects of Delta-8-Estrone Sulfate in Postmenopausal Women.*, J Soc Gynecol Investig. May-Jun;5(3):156-60.

10. Bhavnani BR, Woolever CA, Benoit H, Wong T., 1983, *Pharmacokinetics of Equilin and Equilin Sulfate in Normal Postmenopausal Women and Men.*, J Clin Endocrinol Metab. May;56(5):1048-56.

11. Office of Inspector General. 1997 *Review of the Food and Drug Administration's Handling of Issues Related to Conjugated Estrogens (A-15-96-50002)*

12. Premarin Prescribing Information (2003)

Acta Endocrinologica 1982, 101 592–596

Oestrogens, gonadotrophins and SHBG during oral and cutaneous administration of oestradiol-17β to menopausal women

Lars Fåhraeus and Ulf Larsson-Cohn

Department of Obstetrics and Gynaecology, University Hospital, Linköping, Sweden

Abstract Thirty-eight post-menopausal women were randomly allocated to 6 months of treatment with either 2–4 mg of micronized oestradiol-17β taken orally or 3 mg of oestradiol-17β applied cutaneously. The plasma concentrations of oestrone, oestradiol, LH, FSH and sex hormone binding globulin (SHBG) were determined twice before and after 2, 4 and 6 months of treatment.

In both groups the clinical effects were satisfactory. During treatment the mean oestradiol levels showed similar increases in the two groups while the oestrone concentration was markedly raised only among those taking oestradiol orally. The mean LH and FSH concentrations were significantly lowered in both groups. SHBG was increased with both treatments although more marked in the group on oral medication. Doubling of the oral dose from 2 mg to 4 mg gave significant changes of the LH, FSH and oestrone concentrations.

Thus, in the given doses, the two routes of administration seemed to have similar effects on post-menopausal symptoms and on the plasma concentrations of gonadotrophins and oestradiol. However the plasma oestrone and SHBG levels became significantly higher during the oral than during the cutaneous treatment

Oestrogens are commonly used for relief of climacteric vasomotor symptoms. The hormones are usually given orally but have also been administered intramuscularly or vaginally. One reason for non-oral administration is to avoid the first liver passage of the drug. This should diminish the influences on various liver enzyme systems and thus reduce the metabolic side-effects of oral administration (Larsson-Cohn 1982). Cutaneously applied oestradiol elevates the plasma levels of oestrogens (Whitehead et al. 1980; Lyrenas et al 1981). Therefore cutaneous oestradiol is one alternative when oestrogen substitution is indicated.

The aim of the present investigation was to compare the effects in post-menopausal women of oral and cutaneous administration of oestradiol on the plasma levels of oestrone, oestradiol, LH, FSH and sex hormone binding globulin (SHBG).

Material and Methods

Subjects

Thirty-eight post-menopausal women seeking medical advice for climacteric symptoms agreed to participate in the study. All had LH and FSH levels in the post-menopausal range and none had taken any hormonal preparation during the 3 months prior to the study. Three women had been hysterectomized and one had been bilaterally oophorectomized several years before the start of the investigation. All other women had experienced a natural menopause. The median age of the participants was 52 years (range 42–58 years).

Before and during the trial 7 of the women were treated with one or two of the following drugs: tricyclic antidepressive drugs, analgetics, thiazides or beta-blocking agents. None of the other 31 subjects took any drug regularly and all considered themselves healthy. No participant had clinical or laboratory signs of endocrine, hepatic or renal disease. All women gave their informed consent to the study which was approved by the Ethical Committee of this hospital.

The women were randomly allocated to 6 months of cyclical treatment either with micronized oestradiol-17β

Reprints requests: L. Fåhraeus, Dept. of Obstetrics and Gynaecology, University Hospital, S-58185 Linköping, Sweden

(Östradiol, NOVO A/S, Copenhagen, Denmark) taken orally or with cutaneously administered oestradiol-17β (Oestrogel Laboratoires Besins Iscovesco, Paris, France). The daily oral dose was 2 mg during the first 4 months, whereafter the dose was increased to 4 mg. Cutaneous treatment was given with 5 g of a gel containing 3 mg of oestradiol. The gel was applicated over an abdominal area of about 750 cm². The patients wee instructed to take their oestrogen at bedtime. Every fourth week was treatment-free. Three subject on oral treatment were excluded from the study after 4 months as they did not agree to increase the oestrogen dose to 4 mg and one woman withdrew from the trial after 2 months because of insufficient clinical effects of the medication. In the cutaneous group 2 patients left the study after 2 and 4 months, respectively, as they found the method inconvenient.

Methods

After the patients had fasted overnight blood samples were taken on two separate occasions before treatment and on the 21st or 22nd day of treatment cycles 2, 4 and 6. The median time interval between the two pre-treatment samplings was 2.5 days. The blood was drawn in the morning i.e. 10–12 h after the latest drug administration. After centrifugation serum was stored at −18°C until analyzed.

The serum levels of oestrone and oestradiol were measured with a radioimmunological technique without prior purification (Edqvist & Johansson 1972, Axelsson et al. 1978). In the radioimmunoassay of oestradiol the cross-reaction with oestrone was 10 per cent. The limit of sensitivity for oestrone and oestradiol was 90 pmol/l.

The serum concentration of SHBG was determined as dihydrotestosterone binding capacity as described by Rosner (1972). The limit of sensitivity was 10 nmol/l.

Serum concentrations of LH and FSH were determined by double antibody radioimmunoassay (Greenwood et al. 1963) as described in an earlier paper (Larsson-Cohn et al. 1977).

The oestrone, oestradiol and SHBG analyses were performed by E. D. B. Johansson, Uppsala and the determinations of LH and FSH by B. Kågedal, Department of Clinical Chemistry.

The participants were interviewed about their symptomatology by one of us (L. F.) before treatment and after 2, 4 and 6 months of medication.

Statistics

Oestrone, oestradiol and SHBG levels below the limit of sensitivity were considered zero. The significances of differences during treatment within groups were judged by the Wilcoxon rank sum test and by the paired *t*-test. Comparisons between the groups were judged by the Mann-Whitney U test. The statistical calculations were performed by a computer employing the SPSS-system (Statistical Package for the Social Sciences, University of Pittsburgh).

Table 1

Oestrone and oestradiol levels (pmol/l) in serum before and during treatment with oral and cutaneous oestradiol-17β

			Pre-treatment	Months of medication		
				2	4	6
Oestrone	oral	mean ± SD	243 ± 136	2782 ± 3097***	1988 ± 1111***	3688 ± 2539***
		median	205	2000***	1950***	3750***
	cut	mean ± SD	211 ± 132	791 ± 599***	647 ± 448***	616 ± 266***
		median	158	675***	463***	550***
Oestradiol	oral	mean ± SD	191 ± 164	563 ± 361***	435 ± 283**	601 ± 362**
		median	166	463***	520**	530*
	cut	mean ± SD	178 ± 241	643 ± 471***	555 ± 431**	534 ± 417**
		median	115	495***	348**	400**
E_1/E_2 ratio	oral	mean ± SD	1.10 ± 0.25	3.83 ± 1.90***	4.03 ± 2.13***	5.32 ± 2.76***
		median	0.98	3.45**	3.33**	4.14**
	cut	mean ± SD	1.26 ± 0.65	1.32 ± 0.44	1.27 ± 0.34	1.32 ± 0.45
		median	1.00	1.24	1.29	1.26

E_1 = oestrone, E_2 = oestradiol.

The significances of the mean differences after 2, 4 and 6 months of medication were compared to the mean of the two pre-treatment determinations and were tested by the paired *t*-test. The differences were also tested by the Wilcoxon rank sum test and these results are presented in connection with the median values.

Symbols: * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$

Table 2

LH and FSH levels (IU/l) and SHBG levels (nmol/l) in serum before and during treatment with oral and cutaneous oestradiol-17β.

			Pre-treatment	Months of medication		
				2	4	6
LH	oral	mean ± SD	13.1 ± 3.8	10.8 ± 4.5*	12.1 ± 5.0	9.6 ± 3.1***
		median	12.5	9.5*	11.0*	9.7***
	cut.	mean ± SD	14.4 ± 4.7	10.1 ± 3.9**	10.9 ± 4.0**	11.6 ± 3.6**
		median	13.5	9.3**	11.0**	12.0*
FSH	oral	mean ± SD	44.8 ± 18.5	27.5 ± 15.5***	30.4 ± 13.5***	24.7 ± 10.9***
		median	42.0	25.5***	29.5***	22.3***
	cut	mean ± SD	48.2 ± 19.6	27.8 ± 16.4***	30.1 ± 15.0***	30.8 ± 14.1***
		median	51.0	24.0***	25.0***	27.0**
SHBG	oral	mean ± SD	28.6 ± 13.4	37.0 ± 22.7	49.5 ± 14.2***	55.1 ± 25.0**
		median	29.0	38.0	49.5***	60.0**
	cut	mean ± SD	20.4 ± 15.8	28.1 ± 14.1	30.9 ± 16.5*	31.7 ± 18.1**
		median	17.5	28.5*	30.0**	34.0**

Signs and symbols as in Table 1

Results

Clinical effects

The main complaints of the participants before treatment were hot flushes and sweats. All women except three were either free of symptoms or had their symptoms significantly reduced at all treatment intervals. Two women in the oral group, of whom one withdrew from the study, and one woman in the cutaneous group reported inadequacy of clinical effects at the 2 months control. Before treatment the mean menopausal index of Kupperman (Kupperman et al. 1959) was 23 in the oral and 22 in the cutaneous group. After, respectively, 2, 4 and 6 months the mean menopausal indexes were 12, 9 and 6 in the oral and 9.5 and 4 in the cutaneous group. The indexes were significantly reduced ($P < 0.001$) in both groups at all treatment intervals. No significant differences between the groups were observed.

Oestrogens (Table 1)

Before medication the mean oestrone levels in the two treatment groups were comparable. In the oral group the oestrone increased about 10-fold with the lower dose and 15-fold with the higher dose. In the group on cutaneous oestradiol the mean oestrone concentration rose 3–4-fold. During medication the oral group had significantly higher oestrone concentration ($P < 0.001$) than that on cutaneous treatment.

The mean plasma oestradiol concentration increased 2–3-fold in both groups. The oestradiol levels in the two groups did not differ before or during medication.

As a consequence of the pronounced rise of oestrone in the women on oral treatment the mean oestrone/oestradiol ratio increased from 1.10 to 5.32 at the 6 months control while the ratio remained essentially unchanged in the subjects on cutaneous oestradiol.

SHBG (Table 2)

The mean pre-treatment concentrations of SHBG were not significantly different between the two groups. During medication the SHBG levels rose in both groups. However, during treatment the SHBG levels were significantly higher in the oral than in the cutaneous group.

Gonadotrophins (Table 2)

The mean pre-treatment concentrations of LH and FSH did not differ between the groups. During medication the mean concentrations of LH and FSH were significantly and comparably lowered

after applying oestradiol by either route. All women, except one in each group, had during treatment plasma gonadotrophin levels above the range for fertile women.

The effects of the increment of the oral dose

The raise of the oral oestradiol dose from 2 mg to 4 mg daily resulted in significant alterations of the oestrone, LH and FSH concentrations but not in the levels of SHBG and oestradiol.

Discussion

Adequate doses of oestrogens have good clinical effects on vasomotor symptoms e.g. hot flushes and sweats (Utian 1972; Campbell 1976). Although it has been found in short time studies that even a placebo may be beneficial (Utian 1972; Campbell 1976; Albrecht et al. 1981) oestrogens are more effective (Campbell 1976). The clinical response seems to be dose dependent (Utian 1972). In the present study hot flushes and sweats were the main pre-treatment symptoms. With few exceptions the participants of both treatment groups were effectively relieved from their complaints and it was not possible to establish any significant difference in clinical effectiveness between the two treatments.

The major part of oral oestradiol is converted to oestrone in the gastrointestinal mucosa (Ryan & Engel 1953). This results in higher plasma oestrone than oestradiol levels (Yen et al. 1975; Lyrenäs et al. 1981) which was verified in the present study. The mean serum oestrone concentration became considerably higher than during the ovulatory menstrual cycle (Guerrero et al. 1976). The moderately elevated oestrone levels during the cutaneous treatment were in sharp contrast to those in the oral group. The clinical significance of such high oestrone levels is unknown.

The mean serum levels of oestradiol 10–12 h after the hormone administration were similar in the two treatment groups at all sampling occasions. As oestradiol has higher affinity than oestrone to oestrogen receptors (Whitehead et al. 1981) it is difficult to determine which of the two groups was subject to the greatest total oestrogen stimulation.

The SHBG capacity reacts differently to different sex steroids (Anderson 1974) and has been used as an index of the oestrogenicity of sex hormone therapy (Pogmore & Jequier 1979; Odlind et al. 1980; Larsson-Cohn et al. 1981). In this trial both the oral and the cutaneous oestradiol raised the SHBG although the oral route gave a more pronounced increase than the cutaneous administration. The different effects on the SHBG levels might be explained by the fact that the rise of this plasma protein probably is a consequence of oestrogen induced changes in the liver function. Therefore cutaneous oestradiol which reaches the peripheral circulation without first passing the liver probably induces less SHBG synthesis than does the oral route. This is in agreement with our recent observation that cutaneous compared to oral oestradiol has less influence on the synthesis of other plasma proteins (unpublished data).

Post-menopausal oestrogen treatment depresses the serum gonadotrophins although with commonly used doses LH and FSH usually do not reach the pre-menopausal range (Bolton et al. 1975; Larsson-Cohn et al. 1977; Utian et al. 1978). The results of the present study confirmed these observations. The fact that 4 mg of oestradiol depressed the gonadotrophins more than 2 mg agrees with the results of other investigations showing that the reduction of LH and FSH depends on the potency of the given oestrogen (Bolton et al. 1975; Larsson-Cohn et al. 1977; Utian et al. 1978). The mean reductions of gonadotrophins in the two groups were not different at any treatment interval. This suggests that 3 mg of cutanenous oestradiol is at least as potent as 2 mg of oral oestradiol.

Acknowledgments

This study was supported in part by Laboratoires Besins Iscovesco, Paris and by NOVO A/S, Copenhagen

References

Albrect B H, Schiff I, Tulchinsky D & Ryan K J (1981) Objective evidence that placebo and oral medroxyprogesterone acetate therapy diminish menopausal vasomotor flushes. Am J Obstet Gynecol 139: 631–635.

Anderson D C (1974): Sex-hormone-binding-globulin Clin Endocrinol (Oxf) 3: 69–96.

Axelsson O, Englund D E, Luukkainen T & Johansson E D B (1978): A radioimmunoassay of oestrone in plasma. Plasma levels of oestrone and oestradiol in oophorectomized rhesus monkeys during treatment with subcutaneous implants containing oestrone Acta Endocrinol (Copenh) 87: 609–616

Bolton C. H, Ellwood M, Hartog M, Martin R, Rowe A S & Wensley R T (1975) Comparison of the effects of ethinyl oestradiol and conjugated equine oestrogens in oophorectomized women Clin Endocrinol (Oxf) 4 131–138

Campbell S (1976) Double blind psychometric studies on the effects of natural estrogens on post-menopausal women In Cambell S (ed). The Management of the Menopause and Post-menopausal Years, p 149–158. University Park Press, Baltimore

Edqvist L. E & Johansson E D B (1972): Radioimmunoassay of oestrone and oestradiol in human and bovine peripheral plasma Acta Endocrinol (Copenh) 71 716–730

Greenwood F C, Hunter W M & Glower J S (1963): The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem J 89 114–124

Guerrero R, Aso T, Brenner P F, Cekan Z, Landgren B-M Hagenfeldt K & Diczfalusy E (1976) Studies on the pattern of circulating steroids in the normal menstrual cycle Acta Endocrinol (Copenh) 81: 133–149

Kupperman H S, Wetchler B B & Blatt M H G (1959): Contemporary therapy of the menopausal syndrome JAMA 171 1627–1632.

Larsson-Cohn U (1982): Some biochemical consequences of postmenopausal hormone replacement treatment In van Keep P, Utian W H & Vermeulen A (eds) The Controversial Climacteric, p 29–37 MTP Press Limited, Lancaster

Larsson-Cohn U, Johansson E D B, Kågedal B & Wallentin L (1977) Serum FSH, LH and oestrone levels in postmenopausal patients on oestrogen therapy. Br J Obstet Gynaecol 85 367–372

Larsson-Cohn U, Fåhraeus L, Wallentin L & Zador G (1981) Lipoprotein changes may be minimized by proper composition of a combined oral contraceptive. Fertil Steril 35: 172–179.

Lyrenäs S, Carlstrom K, Bäckström T & von Schoultz B (1981) A comparison of serum oestrogen levels after percutaneous and oral administration of oestradiol-17β Br J Obstet Gynaecol 88: 181–187.

Odlind V, Weiner E, Victor A & Johansson E D B (1980): Effects of sex hormone binding globulin of different oral contraceptives containing norethisterone and lynestrenol Br J Obstet Gynaecol 87: 416–42[illegible]

Pogmore J R & Jequier A M (1979) Sex hormone binding globulin capacity and postmenopausal hormone replacement therapy Br J Obstet Gynaecol 86 568–571.

Rosner W A (1972): A simplified method for the quantitative determination of testosterone-estradiol binding globulin activity in human plasma J Clin Endocrinol Metab 34: 983–988.

Ryan K J & Engel L L (1953). The interconversion of estrone and estradiol by human tissue slices Endocrinology 52: 287–291

Utian W H (1972): The true clinical features of postmenopause and oophorectomy, and their response to oestrogen therapy. S Afr Med J 46 732–737

Utian W H, Katz M, Davey D A & Carr P J (1978) Effect of premenopausal castration and incremental dosages of conjugated equine estrogens on plasma follicle-stimulating hormone, luteinizing hormone, and estradiol Am J Obstet Gynecol 132, 297–302

Whitehead M I, Townsend P T, Kitchin Y, Dyer G, Iqbal M J, Mansfield M D, Young O & Minardi J (1980) Plasma steroid and protein hormone profiles in postmenopausal women following topical administration of oestradiol-17β. In: Mauvais-Jarvis P, Vickers C H F & Wepierre J (eds). Percutaneous Absorption of Steroids, p 231–248. Academic Press, London.

Whitehead M I, Lane G, Dyer G, Townsend P T, Collins W P & King R J B (1981): Oestradiol the predominant intranuclear oestrogen in the endometrium of oestrogen-treated postmenopausal women. Br J Obstet Gynaecol 88: 914–918

Yen S S C, Martin P L, Burnier A M, Czekala N M, Greaney M O Jr & Callantine M R (1975): Circulating estradiol, estrone and gonadotropin levels following the administration of orally active 17β-estradiol in postmenopausal women. J Clin Endocrinol Metab 40 518–521.

Received on February 5th, 1982

Abstract Thirty-e
randomly allocated
oestradiol-17β orally
The concentrations
and phosphollpids w
lipo- otein (HDL)
and the very low de
twice before medica
treatment
Both treatments g
doses increased the
raising the HDL-C/
also increased the I
diol gave only minir
The lipoprotein cha
oral oestradiol mig
disease Therefore
oestradiol treatmer
ficial The cutaneo
able clinical effects
protein pattern

Oestrogen substit
lior..tion of clim
flushes and nigh
be connected w
metrial cancer (J
consequences suc
hydrate and pr
1982) During th

Rep ints requests
Gynaecology, Un
Sweden

ELSEVIER

European Journal of Obstetrics & Gynecology and Reproductive Biology 99 (2001) 222–225

EUROPEAN JOURNAL OF OBSTETRICS & GYNECOLOGY AND REPRODUCTIVE BIOLOGY

www.elsevier.com/locate/ejogrb

Long-term effects of continuous oral and transdermal estrogen replacement therapy on sex hormone binding globulin and free testosterone levels

Ibrahim Serdar Serin[a,*], Bülent Özçelik[a], Mustafa Başbuğ[a], Ercan Aygen[a], Mustafa Kula[b], Ruşen Erez[c]

[a]*Department of Obstetrics and Gynecology, University of Erciyes, 38039 Kayseri, Turkey*
[b]*Department of Nuclear Medicine, University of Erciyes, 38039 Kayseri, Turkey*
[c]*C. Specialist in Statistics, University of Erciyes, 38039, Kayseri, Turkey*

Received 12 January 2001; received in revised form 10 March 2001; accepted 8 April 2001

Abstract

Objective: To determine the long-term effects of estrogen replacement therapy on sex hormone binding globuline (SHBG) and free testosterone (fT) levels in surgical postmenopausal women. *Study Design*: Forty patients with surgical menopause were enrolled in this prospective study. The women were randomly divided into two groups. The first group received oral therapy (continuous conjugated equine estrogens (CEE) — 0.625 mg per day) and the second group received transdermal therapy (patches delivering continuous 17β-estradiol (E2) — 0.05 mg per day). Serum SHBG and fT levels were determined at baseline and after first and second years of treatment. Two-way repeated measures analysis of variance with Bonferroni adjusted post-hoc test and unpaired-*t*-test were performed for statistical analysis with SPSS program. *Results*: Serum SHBG levels increased significantly with oral CEE after first year of treatment ($P < 0.05$) and remained at this level for the next year. Transdermal therapy did not affect SHBG levels after first and second years ($P < 0.05$). Serum fT levels did not change significantly in either group at the end of the first or second years ($P < 0.05$) although there was a significant difference between the groups after 2 years ($P < 0.05$). *Conclusion*: Oral conjugated estrogens increased SHBG levels during therapy. This effect may balance the increased estrogen and androgen stimulation on breast tissue and may be more beneficial to the cardiovascular system in postmenopausal women.

Keywords: Estrogen replacement therapy (ERT); Free testosterone (fT); Sex hormone binding globulin (SHBG)

1. Introduction

Postmenopausal estrogen replacement therapy (ERT) decreases the risk of cardiovascular disease (CVD) and osteoporosis [1,2]. However, recently, circumstantial evidence has indicated a causal relationship between ERT and breast cancer development in long-term users [3,4].

Monitoring some biologic markers which are known to be associated with some risk factors of breast cancer and CVD may be beneficial in women receiving ERT. Sex hormone binding globulin (SHBG) is a plasma glycoprotein with a high binding affinity to free sex steroids particularly free testosterone (fT) and a lower affinity to estradiol [5]. SHBG has shown to be associated with some risk factors of CVD. A protective effect on breast tissue has been found by increasing levels of SHBG and it has also been shown that high levels of biologically active testosterone, free testosterone, is associated with the increased risk of breast cancer and CVD [6–8]. We think that it will be useful to determine these markers which have been found to be related with breast cancer and CVD. It has been shown that ERT increases the estradiol levels [1,2]. However, the long-term effects of ERT on fT and SHBG are as yet unproven. We are unaware of any published study which compares the long-term effects of continuous transdermal and oral ERT on SHBG and fT levels.

This study was designed to assess the effects of transdermal and oral ERT on SHBG and fT levels in surgical menopausal women in a 2-year period.

2. Materials and methods

This study was performed at the Menopause Clinic of Erciyes University Hospital between January 1998 and July

* Corresponding author.
E-mail addresses: sserin@erciyes.edu.tr, serdarserin@hotmail.com (I.S. Serin).

0301-2115/01/$ – see front matter
PII S0301-2115(01)00398-0

Table 1
Sex hormone binding globuline (SHBG) levels (nmol/l) of groups

Groups	N	Baseline	First year	Second year
Oral therapy	20	38.6 ± 29.0	74.4 ± 56.2*	89.3 ± 57.3*,**
Transdermal therapy	20	42.1 ± 23.4	41.1 ± 22.4	44.1 ± 28.1

* Significantly different from baseline (column 4) and transdermal therapy group (columns 4 and 5) ($P < 0.05$).
** Significantly different from baseline ($P < 0.01$).

2000. Our study was approved by the Institutional Review Board of Erciyes University Hospital. Fifty premenopausal patients aged 40–48 (mean 45.0 ± 2.07) undergoing hysterectomy and bilateral salpingo-oophorectomy for benign conditions were enrolled in this prospective study. Each patient was free from any other disorder including hypertension, liver disease, diabetes mellitus, thromboembolism, or history of alcohol abuse or smoking. At the beginning of this study, the patients were randomly assigned into two groups, each consisting of 25 patients. The first group received transdermal estradiol 0.05 mg per day continuously (CIBA Pharmaceutical Co., Summit, NJ), and the second group received continuous oral conjugated estrogen 0.625 mg per day (Premarin; Ayerst Laboratories, New York, NY). ERT was started 1 month after surgery. Seven patients who did not turn up for follow-up visits, and three patients with skin reactions were excluded from the study. A total of 40 patients, 20 in each group completed the study. For ethical reasons, symptomatic women were not included as a control group in our long-term study.

Blood samples were taken at baseline before therapy and after first and second years. Blood samples for fT and SHBG assay were obtained by veinpuncture between 07.30 and 11.00 h after a compulsory 12 h fast, and the plasma was separated and frozen at −70°C until assayed. SHBG (Orion Diagnostica, Espoo, Finland) levels were determined using immunoradiometric assay (IRMA) method and fT (Diagnostic system Laboratories, TX, USA) levels were determined using radioimmunoassay (RIA). The sensitivity and the intra- and inter-assay coefficients of the variation of the assays used were, respectively, as follows: SHBG, 0.5 nmol/l, 4–5.5 and 3.3–6.9%, and free testosterone, 0.18 pg/ml, 3.7–6.2 and 7.3–9.7%.

Two-way repeated measures analysis of variance with Bonferroni adjusted post-hoc test and unpaired-t-test were performed for statistical analyses with SPSS program. The values are expressed as mean ± S.D. $P < 0.05$ was defined as statistically significant.

3. Results

The mean ages were 45.0 ± 2.0 and 44.9 ± 2.1 years, BMI values were 24.8 ± 3.9 and 24.3 ± 3.3 kg/m^2 in the transdermal and oral therapy groups, respectively. There was no significant difference between the two groups in terms of mean age or BMI before therapy ($P < 0.05$).

Table 2
Free testosterone (fT) levels (pg/ml) of groups

Groups	N	Baseline	First year	Second year
Oral therapy	20	1.6 ± 0.8	1.0 ± 0.6	1.0 ± 0.3
Transdermal therapy	20	1.4 ± 0.7	1.1 ± 0.6	1.4 ± 0.6*

* Significantly different from oral therapy group ($P < 0.05$).

The serum SHBG levels of the two groups are shown in Table 1. There was no significant difference in SHBG levels between the groups at the onset of trial ($P < 0.05$), whereas we found a significant difference after the first and second years of treatment ($P < 0.05$). The SHBG levels increased significantly with oral estrogens ($P < 0.05$) after the first year, although no significant difference was found between the first and second year values of SHBG in oral therapy group ($P < 0.05$). Transdermal therapy did not affect SHBG levels in the 2 years ($P < 0.05$).

The fT levels are given in Table 2. While there was no significant difference between the groups before treatment and after the first year of trial ($P < 0.05$), a significant difference was found at the end of the second year of therapy ($P < 0.05$). Changes in fT levels within the groups were not significant after first or second years ($P < 0.05$).

4. Discussion

While use of hormone replacement therapy effectively alleviates menopausal symptoms and prevents osteoporosis and cardiovascular disease, there is concern that it has a detrimental impact on breast cancer risk. Some epidemiologic studies indicate that long-term therapy is associated with a slightly increased risk of developing breast cancer [3,4]. It has also been found that the current use of hormone replacement therapy lowers the efficacy of screening for breast cancer [9]. Recently, The Heart and Estrogen/Progestin Study (HERS) group found no overall effect of 4.1 years of therapy with estrogen plus progestin on secondary prevention of coronary heart disease in postmenopausal women [10]. Because of these different effects, it seems necessary to evaluate the risk/benefit ratio of ERT for each patient individually. The cardioprotective effects of ERT has been shown in several studies, but the long-term effects of ERT on risk factors such as SHBG and fT in cardiovascular system and breast tissue are not well established.

The association of breast cancer and cardiovascular disease with several risk factors such as androgen levels and android obesity has been discussed in several studies [11,12]. Androgens have been hypothesized to increase breast cancer risk, either directly by increasing the growth and proliferation of breast cancer cells, or indirectly by their conversion to estrogens [13–15]. It has also been shown that androgen excess may be a signal of increased risk of coronary artery disease [16].

Android obesity is associated with an increased aromatization of androgens and decreased levels of SHBG, resulting in an increase in free, biologically active, sex steroid concentrations [12,17]. We think that the similar BMI values of patients in both groups at the beginning of our study has eliminated the difference in androgenic aromatization. SHBG has been shown to be associated with some risk factors of cardiovascular disease. It is usually positively correlated to high density lipoprotein cholesterol (HDL-C) levels, and negatively to insulin and triglyceride (TG) levels in postmenopausal women [18,19]. SHBG is an index of androgenism in women and of insulin resistance in both sexes, and might be useful in prediction of cardiovascular risk [20].

Different routes of ERT administration and their effects on cardiovascular system and breast tissue remain to be the subject of investigations. The hepatic metabolism of oral and transdermal compounds is different — oral estrogens produce marked hepatocellular effects, such as increased serum levels of sex hormone binding globuline which could partially counterbalance the unfavorable effects of estrogen stimulation on breast tissue [21]. Although higher estrogen levels may have both beneficial and adverse effects, reducing the levels of endogenous estrogens may be a promising means in the prevention of breast cancer. Increasing levels of SHBG with oral ERT may also have beneficial effects on the cardiovascular system by reducing free testosterone levels. On the other hand, low circulating bioavailable testosterone, androgen deficiency, may cause low libido. Testosterone replacement therapy results in significant improvement in symptomatology and hence the quality of life for majority of women [22].

Transdermal estrogens have only been used for a few years and it is unknown whether this route of administration may produce different effects on breast cancer risk when compared to oral estrogens. However, it is well established that transdermal therapy has cardioprotective effects by lowering triglycerides, and total and LDL cholesterol levels [23,24]. Crook and Stevenson [25] concluded that transdermal monotherapy neither increased nor decreased HDL-C levels. Nieto et al. [26] reported a significant increase in HDL-C levels with continuous transdermal estradiol 0.05 mg per day and dydrogesterone 10 mg per day (days 15–28) after 6 months. It seems that oral dydrogesterone affects the hepatic metabolism which causes favorable changes in HDL-C levels. We were not able to find any study which evaluates the long-term effects of transdermal estradiol on hepatic proteins, especially SHBG levels.

In this study, our aim was to assess the long-term effects of ERT on SHBG and fT levels which were found to be risk factors of breast cancer and CVD in surgical menopausal women. In a study carried out at our institution, Başbuğ et al. [27] reported that oral conjugated estrogens increased SHBG levels in 22 weeks, whereas transdermal estrogen had no effect. Obel et al. [28] determined a significant increase in SHBG levels with oral 2 mg oestradiol and 1 mg norethisterone acetate (NETA) in combined and sequential therapy. In the study of Pedersen and Jensen [29], SHBG and lipid levels were shown not to change with transdermal 0.05 mg per day twice a week plus 10 mg oral medroxyprogesterone acetate (days 12–25) after 1 year.

In our study, SHBG levels increased with oral therapy at the first year which confirms other studies and these levels remained the same after second year of treatment. Transdermal therapy had no effect on SHBG levels after first or second years. It seems that duration of transdermal therapy does not affect hepatic metabolism. While fT levels did not change significantly within the groups, there was a significant difference in fT levels between the groups after 2 years. We think that it may be possible to describe this difference between oral and transdermal therapy with long-term studies. Further studies are needed to determine the relationship between ERT and risk factors of breast cancer and CVD.

In conclusion, increasing levels of SHBG with oral therapy may have a balancing effect on breast tissue and this treatment may also be more beneficial to the cardiovascular system.

References

[1] Grady D, Rubin SM, Petitti DB, Fox CS, Black D, Ettinger B, Ernster VL, Cummings SR. Hormone therapy to prevent disease and prolong life in postmenopausal women. Ann Int Med 1992;117:1016–37.

[2] Stamper MJ, Colditz GA, Willett WC, Manson JE, Rosner B, Speizer FE, et al. Postmenopausal estrogen therapy and cardiovascular disease. Ten-year follow-up from the Nurses Health Study. N Engl J Med 1991;325:756–62.

[3] Colditz GA. Relationship between estrogen levels, use of hormone replacement therapy, and breast cancer. J Natl Cancer Instit 1998;90:814–23.

[4] Sillero-Arenas M, Delgado Rodriguez M, Ridigues-Canteras R, Bueno-Cavanillas A, Galvez-Vargas R. Menopausal hormone replacement therapy and breast cancer: meta-analyses. Obstet Gynecol 1992;72:286–94.

[5] Rosner W. The functions of corticosteroid-binding globulin and sex hormone-binding globulin: recent advances. Endocrinol Rev 1990;11:80–91.

[6] Berrino F, Muti P, Micheli A, Bolelli G, Krogh V, Sciajno R, et al. Serum sex hormone levels after menopause and subsequent breast cancer. J Natl Cancer Instit 1996;88:291–96.

[7] Dorgan JF, Longcope C, Stephenson HE, Falk RT, Miller R, Franz C, et al. Relation of prediagnostic serum estrogen and androgen levels to breast cancer risk. Cancer Epidemiol Biomarker Prevent 1996;5:533–39.

[8] Philips GB, Pinkernell BH, Jing TY. Relationship between serum sex hormones and coronary artery disease in postmenopausal women. Arterioscler Throm Vasc Biol 1997;17:695–701

[9]

[10]

[11]

[12]

[13]

[14]

[15]

[16]

[17]

[18]

[19]

[9] Beral V, Banks E, Reeves G, Wallis M. Hormone replacement therapy and high incidence of breast cancer between mammographic screens. Lancet 1997;349:1103–4.

[10] Hulley S, Grady D, Bush T, Furberg C, Herrington D, Riggs B, et al. Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women, vol. 280. Heart and Estrogen/Progestin Replacement Study Research (HERS) Group. JAMA, 1998. p. 605–13.

[11] Tchernof A, Toth MJ, Poehlman ET. Sex hormone binding globulin levels in middle-aged premenopausal women. Associations with visceral obesity and metabolic profile. Diab Care 1999;22:1875–81.

[12] Sellers TA, Kushi LH, Potter JD, Kaye SA, Nelson CL, McGovern PG, et al. Effect of family history, body fat distribution and reproductive factors on the risk of postmenopausal breast cancer. NEJM 1992;326:652–56.

[13] Lipman M, Bolan G, Huff K. The effects of androgens and antiandrogens on hormonoresponsive human breast cancer in long-term tissue culture. Cancer Res 1976;36:4610.

[14] James VH, McNeill JM, Lai LC, Newton CJ, Ghilchick MW, Reed MJ. Aromatase activity in normal breast and breast tumor tissues: in vivo and in vitro studies. Steroids 1987;50:269–79.

[15] Thijssen JH, Blankenstein MA, Danker GH, Daroszewski J. Endogenous steroid hormones and local aromatase activity in the breast. J Steroid Biochem Mol Biol 1991;39:799–804.

[16] Wild RA. Obesity, lipids, cardiovascular risk, and androgen excess. Am J Med 1995;98:27–32.

[17] Folsom AR, Kay SA, Prineas RJ, Potter JD, Gapstur SM, Wallace RB. Increased incidence of carcinoma of the breast associated with abdominal adiposity in postmenopausal women. Am J Epidemiol 1990;131:794–803.

[18] Semmens J, Rouse I, Beilin LJ. Relationship of plasma HDL-cholesterol to testosterone, estradiol and sex hormone-binding globulin levels in men and women. Metabolism 1983;32:428–32.

[19] Haffner SM, Katz MS, Stern MP. Association of decreased sex hormone binding globulin and cardiovascular risk factors. Arteriosclerosis 1989;9:136–43.

[20] Pugeat M, Moulin P, Cousin P, Fimbel S, Nicholas MH, Crave JC. Interrelations between sex hormone-binding globulin (SHBG), plasma lipoproteins and cardiovascular risk. J Steroid Biochem Mol Biol 1995;53:567–72.

[21] Campagnoli C, Biglia N, Altare F, Lanza MG, Lesca L, Cantamessa C, Peris C, et al. Differential effects of oral conjugated estrogens and transdermal estradiol on insulin-like growth factor I, growth hormone and sex hormone binding globulin serum levels. Gynecol Endocrinol 1993;7:251–8.

[22] Davis S. Androgen replacement in women: a commentary. J Clin Endocrinol Metab 1999;84:1886–91.

[23] Whitecroft SI, Crook DI, Marsh MS, Ellerington MC, Whitehead MI, Stevenson JC. Long-term effects of oral and transdermal hormone replacement therapies on serum lipid and lipoprotein concentrations. Obstet Gynecol 1994;84:222–6.

[24] Mattsson LA, Samsioe G, Scoultz B, Livebrant M, Wiklund I. Transdermally administered oestradiol combined with oral medroxyprogesterone acetate: the effects on lipoprotein metabolism in postmenopausal women. Br J Obstet Gynaecol 1993;100:450–3.

[25] Crook D, Stevenson SC. Transdermal hormone replacemant therapy, serum lipids and lipoprotein. Br J Clin Pract 1996;86:17–21.

[26] Nieto JJ, Cogswell D, Jesinger D, Hardiman P. Lipid effects of hormone replacement therapy with sequential transdermal 17β-estradiol and oral dydrogesterone. Obstet Gynecol 2000;95:111–4.

[27] Basbug M, Aygen E, Tayyar M, Muhtaroglu S, Demir I, Okten S. Twenty two weeks of estradiol increases sex hormone-binding globulin in surgical menopausal women. Eur J Obstet Gynecol Reprod Biol 1997;73:149–52.

[28] Obel EB, Jensen M, Svenstrup B, Bennett P, Micic S, Nielsen H. A two-year double-blind controlled study of the clinical effect of combined and sequential postmenopausal replacement therapy and steroid metabolism during treatment. Maturitas 1993;16:13–21.

[29] Pedersen OD, Jensen HK. Long-term treatment with transcutaneous estradiol and oral medroxyprogesterone acetate. Acta Obstet Gynecol Scand 1992;71:593–8.

Comparison of Estrogen and Androgen Levels After Oral Estrogen Replacement Therapy

Cristin C. Slater, M.D., Chunying Zhang, M.D., Howard N. Hodis, M.D., Wendy J. Mack, Ph.D., Robert Boostanfar, M.D., Donna Shoupe, M.D., Richard J. Paulson, M.D., and Frank Z. Stanczyk, Ph.D.

OBJECTIVE: To assess the extent of accumulation of circulating estrone (E_1), total and free estradiol (E_2) and estrone sulfate (E_1S) levels in postmenopausal women receiving prolonged oral E_2 therapy and to determine the effect of increased estrogenicity on free testosterone levels.

STUDY DESIGN: Descriptive study involving 14 healthy postmenopausal women during a three-year period. Group 1 (n=7) took a placebo. Group 2 (n=7) took 1 mg micronized E_2 daily. Blood samples were taken at one, two and three years. E_2, E_1 and total testosterone were quantified by radioimmunoassay (RIA) following extraction and celite chromatography. Free testosterone and E_2 were calculated. Sex hormone–binding globulin (SHBG) and E_1S were quantified by RIA.

RESULTS: In the control group, none of the hormone levels changed significantly. Free testosterone decreased 49% in women taking E_2 replacement as compared to a 7% decline in women taking placebo. In women taking E_2 replacement, E_1, E_2, E_1S, free E_2 and SHBG levels increased 10, 6, 51, 2 and 2 times, respectively, between baseline and year 3.

Routine estrogen replacement substantially decreases free testosterone (49% decrease).

CONCLUSION: E_1, E_2 and E_1S levels significantly increased with E_2 replacement. Free testosterone levels decreased with E_2 replacement. Testosterone replacement may be warranted when giving postmenopausal women estrogen replacement therapy. (J Reprod Med 2001;46:1052–1056)

Keywords: androgens, estrogens, menopause, estrogen replacement therapy, hormone replacement therapy.

From the Department of Obstetrics and Gynecology; Atherosclerosis Research Unit, Division of Cardiology; and Department of Preventive Medicine, University of Southern California Keck School of Medicine, Los Angeles.

Dr. Slater is Assistant Professor, Department of Obstetrics and Gynecology.

Dr. Zhang is Research Associate, Department of Obstetrics and Gynecology.

Dr. Hodis is Associate Professor, Department of Medicine and Preventive Medicine, and Director, Atherosclerosis Research Unit, Division of Cardiology.

Dr. Mack is Associate Professor, Department of Preventive Medicine.

Dr. Boostanfar is Clinical Instructor, Department of Obstetrics and Gynecology.

Drs. Shoupe and Paulson are Professors, Department of Obstetrics and Gynecology.

Dr. Stanczyk is Professor, Department of Obstetrics and Gynecology.

Funded in part by National Institutes of Health Research Grant U01/HL49298, National Heart, Lung and Blood Institute, and Office of Research on Minority Health, National Institutes of Health.

Address correspondence to: Cristin C. Slater, M.D., Department of Obstetrics and Gynecology, Women's and Children's Hospital, 1240 North Mission Road, Room 8K9, Los Angeles, CA 90033 (coulam@hsc.usc.edu).

Financial Disclosure: The authors have no connection to any companies or products mentioned in this article.

0024-7758/01/4612-1052/$15.00/0

Introduction

Oral estrogens have been utilized for estrogen replacement therapy (ERT) for many years by millions of postmenopausal women (PMW). Much is known about the beneficial effects of estrogen, including its beneficial effects of decreasing cardiovascular disease,[1,2] osteoporosis,[3] genitourinary symptoms[4] and senile dementia.[5] Despite this vast knowledge of estrogenic effects of ERT, relatively little is known about the effect of long-standing use of ERT on the estrogenic-androgenic milieu. We recently reported that during one and one-half years of treatment with oral estradiol (E_2) in PMW, there is accumulation of serum levels of E_2 and estrone sulfate (E_1S).[6] Oral estrogens elevate sex hormone–binding globulin levels (SHBG), causing decreased free testosterone levels. Thus, increased estrogenicity during years of oral ERT may lead to a state of bioavailable testosterone deficiency.

Possible effects of decreased testosterone include osteoporosis, muscle wasting, anergy, decreased libido, decreased sense of well-being and depression. There is promise of an array of androgen supplements as therapy adjuvant to traditional ERT. These preparations aim to replace the androgen loss that is due to either menopause itself and/or decreases in androgens resultant to ERT effects.

Due to the lack of data regarding long-term menopausal ERT on ovarian steroid levels, the objective of the present study was: (1) to assess the extent of accumulation of circulating estrone (E_1), total and free E_2, and E_1S levels in PMW receiving prolonged oral E_2 therapy, and (2) to determine the effect of increased estrogenicity on total and free testosterone levels.

Methods

Fourteen PMWs between the ages of 52 and 67 were studied. Menopause was defined as not having menses for greater than one year and a follicle-stimulating hormone level >40 mIU/mL. All women were healthy, with a body mass index of <28 kg/m^2, and had undergone hysterectomy. None of the women had a history of polycystic ovary syndrome. This was a prospective study performed in a university hospital setting. Group 1 ($n=7$) took a placebo. Group 2 ($n=7$) took 1 mg of micronized E_2 daily. Blood samples were taken at one, two and three years. E_2, E_1 and total testosterone were quantified by radioimmunoassay (RIA) following extraction and celite chromatography. Free testosterone and E_2 were calculated.[7] Sex hormone–binding globulin and E_1S were quantified by specific RIA. One of the control subjects and two of the estrogen-treated subjects did not have enough serum aliquoted to measure E_1S. Statistical analysis was done using repeated measure ANOVA and the paired *t* test.

Testosterone replacement via a nonoral route may be a feasible way to improve the above symptoms without affecting liver enzymes and/or lipids.

Results

Table I shows repeated measures analysis of androgen-estrogen balance with group comparisons at each time point (baseline, one year, two years and three years). In the control group, none of the hormone levels changed significantly. Free testosterone deceased 49% ($P=.0003$) in women taking E_2 replacement as compared to a 7% decline in women taking placebo. In women taking E_2 replacement, E_1, E_2, E_1S, free E_2 and SHBG levels increased 10, 6, 51, 2 and 2 times, respectively, between baseline and year 3.

Significant differences between the two groups were found at the following time points: percent change in free testosterone, when compared to baseline, at years 2 ($P=.04$) and 3 ($P=.006$); E_1 at years 1 ($P=.002$), 2 ($P=.008$) and 3 ($P=.009$); E_2 at years 1 ($P=.004$), 2 ($P=.001$) and 3 ($P=.005$); free E_2 at year 1 ($P=.05$); and SHBG at years 2 ($P=.02$) and 3 ($P=.005$).

Discussion

Menopause has been associated with a decline in Δ^5 androgens, dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS). The Δ^4 androgens have not been found to decrease with menopause.[8,9] The effect of noncontraceptive estrogen on levels of androgen and other steroid hormones has been studied with a variety of designs, age groups and estrogen preparations, usually in women who were not well characterized regarding determinants of hormone status. Results vary from no significant change[10] to decreased[11] or increased[12] levels of androgens by estrogen use. Pat-

Table I *Repeated Measures Analysis of Androgen-Estrogen Balance (Group Comparisons at Each Time Point)*

		Baseline		1 Yr		2 Yr		3 Yr		
Hormone		Mean	SD	Mean	SD	Mean	SD	Mean	SD	n
Total	Control	23.4	6.9	41.0	10.2	32.0	8.3	38.3	12.3	7
testosterone	Estrogen	19.3	4.9	20.1	3.6	28.1	6.8	21.9	5.8	7
(ng/dL)	P group[a]	0.63		0.09		0.73		0.25		
Free	Control	1.84	0.60	2.59	1.25	1.81	0.50	2.15	0.74	7
testosterone	Estrogen	2.40	0.83	1.84	0.27	1.67	0.38	1.55	0.35	7
(pg/mL)	P group	0.56		0.10		0.60		0.08		
Free	Control	—	—	0.07	0.21	−0.05	0.15	−0.07	0.13	7
testosterone[b]	Estrogen	—	—	−0.30	0.05	−0.39	0.07	−0.49	0.05	7
(%)	P group	—		0.08		0.04		0.006		
E_1 (pg/mL)	Control	43.7	7.0	50.0	7.0	45.9	9.6	52.6	11.3	7
	Estrogen	38.3	4.8	271.6	40.7	220.3	30.2	396.1	91.5	7
	P group	0.53		0.002		0.0008		0.009		
E_2 (pg/mL)	Control	21.3	4.7	19.7	2.9	18.0	2.9	18.4	2.3	7
	Estrogen	12.7	1.0	67.4	10.6	60.9	7.8	76.6	13.5	7
	P group	0.13		0.004		0.001		0.005		
E_1S (pg/mL)	Control	0.26	0.09	0.28	0.09	0.26	0.08	0.29	0.12	6
	Estrogen	0.26	0.10	4.97	2.17	4.36	1.81	13.4	5.07	5
	P group	0.96		0.08		0.07		0.06		
Free E_2	Control	3.03	1.11	2.23	0.49	1.75	0.58	2.32	0.65	7
(pg/mL)	Estrogen	1.67	0.29	4.09	0.70	3.01	0.35	4.10	0.60	7
	P group	0.28		0.05		0.08		0.07		
SHBG	Control	27.1	4.6	35.5	9.6	35.6	7.7	30.6	4.7	7
(nmol/L)	Estrogen	25.3	6.1	50.1	6.1	59.7	4.6	54.1	4.9	7
	P group	0.82		0.22		0.02		0.005		

[a]*t* Test was used.
[b]Percent change in free testosterone as compared to baseline.
P = placebo.

terns of alterations may contribute to understanding of the pathways of steroid metabolism, such as the effects of estrogens on adrenal cortical function and the hypothalamic-pituitary-ovarian axis.

Our study was unique in that it was a long-term one, evaluating ovarian steroid levels over a three-year period. Our results show that routine estrogen replacement substantially decreases free testosterone (49% decrease). This may be due to increased SHBG levels, therefore decreasing free testosterone levels. Our data did not show a decrease in total testosterone levels. However, a prior prospective, randomized study by Casson et al did show a decrease in both total and free testosterone with physiologic estrogen replacement[13] and attributed this possible menopausal decrease in total testosterone levels with physiologic estrogen replacement to decreased gonadotropin drive.

Although the differences in free testosterone between the two groups were not statistically significant, the differences were substantial. Statistical significance is difficult to achieve with a small sample size.

The state of bioavailable testosterone deficiency may further the accelerate osteoporosis and muscle wasting that naturally occur with aging. Menopausal symptoms, such as loss of libido, anergy and sense of well-being, may be exacerbated. In contrast, lipids and insulin sensitivity may benefit from decreased free testosterone levels. Testosterone replacement via a nonoral route may be a feasible way to improve the above symptoms without affecting liver enzymes and/or lipids. This is due to the avoidance of the "first-pass effect" through the hepatic circulation.

Not surprisingly, all of the estrogen levels increased with micronized E_2 replacement. E_1, E_2, free E_2 and E_1S increased 934%, 503%, 145% and 5,053%, respectively. Sex hormone–binding globulin increased 113%. E_1S increased 10% more than E_2 with micronized E_2 replacement. If conjugated equine estrogens had been used for replacement, there may have been a much larger increase in E_1S levels. A prior study confirmed this large accumulation of E_1S with long-term oral estrogen replacement.[6]

Published data show that menopausal ERT also further decreases DHEA and DHEAS levels. The declining DHEAS levels may suggest a direct adrenal effect of estrogen.[13] Natural aging is already associated with a decline in DHEAS. The implications of a further decline in DHEA, DHEAS and free testosterone levels due to ERT are unknown at this time. Although androgen replacement therapy is available, long-term trials have not been done. Most of the published literature on androgen replacement is on oral methyltestosterone. Comparisons of oral estrogen-androgen and estrogen-only therapy have shown that combined treatment with estrogen-androgen therapy has greater efficacy on vasomotor symptoms when compared to estrogen-only therapy. Simon et al[14] also found that estrogen-only treatment significantly increased SHBG levels ($P < .01$), whereas decreases occurred with estrogen-androgen treatment ($P < .006$).

Watts et al[15] compared oral esterified estrogen replacement with combined oral esterified estrogen and methyltestosterone treatment on bone mineral density, menopausal symptoms and lipid-lipoprotein profiles in surgically menopausal women. Both treatment regimens prevented bone loss at the spine and hip. However, combined estrogen-androgen therapy was associated with a significant increase in bone mineral density as compared to baseline ($P < .01$). In the estrogen group, high-density lipoprotein (HDL) cholesterol increased significantly, and low-density lipoprotein cholesterol decreased significantly. Cholesterol, HDL cholesterol and triglycerides decreased significantly in the estrogen-androgen group. Menopausal symptoms of somatic origin (hot flashes, vaginal dryness and insomnia) were improved significantly in both groups.

Raisz et al[16] compared the effects of estrogen alone (1.25 mg conjugated equine estrogen) to combined estrogen + androgen therapy (1.25 mg esterified estrogen + 2.5 mg methyltestosterone) on biochemical markers of bone formation and resorption in PMW. Both groups showed a similar decrease in urinary excretion of the bone resorption markers, deoxypyridinoline, pyridinoline and hydroxyproline. Patients treated with estrogen only showed decreases in the serum markers of bone formation, bone-specific alkaline phosphatase, osteocalcin and C-terminal procollagen peptide. In contrast, subjects treated with estrogen-androgen showed increases in these markers of bone formation. Thus, estrogen-androgen therapy is a potentially beneficial hormone replacement regimen in osteoporotic women. Long-term studies are needed to determine the relative benefits and risks of combined estrogen-androgen treatment regimens. Little is known about other forms of testosterone or other routes of delivery, such as transdermal, sublingual and transvaginal. These alternate routes of delivery bypass the hepatic circulation and may be safer for long-term testosterone replacement.

Shifren et al[17] evaluated the effects of 12 weeks of transdermal testosterone (150 and 300 μg/d) in women who had impaired sexual function after surgically induced menopause. Transdermal testosterone was found to improve sexual function and psychological well-being in these women. The 300 μg/d dose resulted in statistically significant increase in scores for frequency of sexual activity and pleasure-orgasm in the Brief Index of Sexual Functioning for Women ($P = .03$ for both comparisons with placebo). The positive well-being, depressed mood and composite scores of the Psychological General Well-Being Index also improved significantly with the 300 μg/d testosterone dose ($P = .04$, .03 and .04, respectively, for the comparison with placebo). Thus, transdermal testosterone replacement may be indicated for women with low testosterone concentrations and impaired sexual function.

While it is commonly accepted to prescribe androgen replacement to surgically castrated women, postmenopausal androgen replacement remains under study at this time. The benefits of menopausal combined estrogen-androgen replacement must be evaluated in conjunction with possible long-term risks before menopausal androgen replacement therapy is universally prescribed.

References

1. Paganini-Hill A, Ross RK, Henderson BE: Postmenopausal oestrogen treatment and stroke: A prospective study. Br Med J 1988;297:519–522
2. Stampfer MJ, Colditz GA, Willett WC, et al: Postmenopausal estrogen therapy and cardiovascular disease: Ten-year follow-up from the Nurses' Health Study. N Engl J Med 1991; 325:756–762
3. Kiel DP, Felson DT, Anderson JJ, et al: Hip fracture and the use of estrogens in postmenopausal women: The Framingham Study. N Engl J Med 1987;317:1169–1174
4. Raz R, Stamm WE: A controlled trial of intravaginal estriol in postmenopausal women with recurrent urinary tract infections. N Engl J Med 1993;329:753–756
5. Paganini-Hill A, Henderson VW: Estrogen deficiency and risk of Alzheimer's disease in women. Am J Epidemiol 1994;140:256–261

6. Slater C, Hodis H, Mack W, et al: Markedly elevated levels of estrogen sulfate following long-term oral but not transdermal administration of estradiol in postmenopausal women. Menopause (in press)
7. Sodergard R, Backstrom T, Shanbhag V, et al: Calculation of free and bound fractions of testosterone and estradiol-17β to human plasma proteins at body temperature. J Steroid Biochem 1982;16:801–810
8. Meldrum DR, Davidson BJ, Tataryn IV, et al: Changes in circulating steroids with aging in postmenopausal women. Obstet Gynecol 1981;57:624–628
9. Judd HL, Judd GE, Lucas WE, et al: Endocrine function of the postmenopausal ovary: Concentration of androgens and estrogens in ovarian and peripheral vein blood. J Clin Endocrinol Metab 1974;39:1020–1024
10. Rose DP, Fern M, Liskowski L, et al: Effect of treatment with estrogen conjugates on endogenous plasma steroids. Obstet Gynecol 1977;49:80–82
11. Helgason S, Carlstrom K, Damber MG, et al: Effects of various oestrogens on circulating androgens and cortisol during replacement therapy in post-menopausal women. Maturitas 1981;3:301–308
12. Abraham GE, Maroulis GB: Effect of exogenous estrogen on serum pregnenolone, cortisol, and androgens in postmenopausal women. Obstet Gynecol 1975;45:271–274
13. Casson PR, Elkind-Hirsch KE, Buster JE, et al: Effect of postmenopausal estrogen replacement on circulating androgens. Obstet Gynecol 1997;90:995–998
14. Simon J, Klaiber E, Wiita B, et al: Differential effects of estrogen-androgen and estrogen-only therapy on vasomotor symptoms, gonadotropin secretion, and endogenous androgen bioavailability in postmenopausal women. Menopause 1999;6:138–146
15. Watts NB, Notelovitz M, Timmons MC, et al: Comparison of oral estrogens and estrogens plus androgen on bone mineral density, menopausal symptoms, and lipid-lipoprotein profiles in surgical menopause. Obstet Gynecol 1995;85:529–537
16. Raisz LG, Wiita B, Artis A, et al: Comparison of the effects of estrogen alone and estrogen plus androgen on biochemical markers of bone formation and resorption in postmenopausal women. J Clin Endocrinol Metab 1996;81:37–43
17. Shifren JL, Braunstein GD, Simon JA, et al: Transdermal testosterone treatment in women with impaired sexual function after oophorectomy. N Engl J Med 2000;343:682–688

0021-972X/00/$03.00/0
The Journal of Clinical Endocrinology & Metabolism

Vol. 85, No 12
Printed in U S A

Associations among Oral Estrogen Use, Free Testosterone Concentration, and Lean Body Mass among Postmenopausal Women*

BARBARA A. GOWER AND LARA NYMAN

University of Alabama at Birmingham, Department of Nutrition Sciences (B.A.G.), Division of Physiology and Metabolism, and Clinical Nutrition Research Center, and Department of Physiology and Biophysics (L.N.), Birmingham, Alabama 35294-3360

ABSTRACT

Circulating concentrations of sex hormone-binding globulin (SHBG) are increased by use of oral estrogen. The objective of this study was to determine whether postmenopausal women who used oral estrogen had higher serum concentrations of SHBG and lower serum concentrations of free testosterone (T) than nonusers, and whether free T was associated with lean body mass, particularly skeletal muscle mass. Subjects were 70 postmenopausal women, 46–55 yr old, 46 of whom used oral estrogen. Total and regional body composition were determined by dual-energy x-ray absorptiometry. Serum concentrations of SHBG, total T, and estradiol (E_2) were determined by RIA. Free T was calculated from concentrations of total T and SHBG. Hormone users had higher serum concentrations of E_2 and SHBG (182.0 ± 58.5 *vs.* 82.9 ± 41.1 nmol/L, mean ± SD, $P < 0.001$) and lower concentrations of free T (3.7 ± 2.2 *vs.* 7.9 ± 4.1 pmol/L, mean ± SD, $P < 0.001$); total T did not differ. Total lean mass and leg lean mass were significantly correlated with free, but not total T [r values of 0.29 ($P < 0.05$) and 0.31 ($P < 0.01$) for total and leg lean mass, respectively, *vs.* free T]; arm lean mass was not correlated with either measure of T. Serum E_2 was significantly correlated with SHBG ($r = 0.50$, $P < 0.001$) and free T ($r = -0.33$, $P < 0.01$). These observations imply that, by reducing the concentration of bioavailable T, oral estrogen therapy may accelerate or augment lean mass loss among postmenopausal women. This conclusion awaits confirmation by longitudinal observation. (*J Clin Endocrinol Metab* **85**: 4476–4480, 2000)

ORAL ESTROGEN THERAPY, because of the first pass effect, results in exposure of the liver to supraphysiological concentrations of estrogen. One of the consequences of this exposure is an alteration in the production of several hepatic proteins. Concentrations of sex hormone-binding globulin (SHBG) (1) and apolipoprotein A-I (Apo A-I) (2) are higher, and insulin-like growth factor (IGF)-I are lower (3), in women who use oral estrogen therapy, when compared with nonusers.

The physiological ramifications of altered hepatic protein production with estrogen use are not entirely clear. This is particularly true with respect to the elevation in SHBG. SHBG binds to circulating testosterone (T). In premenopausal women, approximately 62% of circulating T is bound to SHBG; another 36–37% is bound to albumin, and approximately 1.5% is unbound (4). It is assumed that the unbound (or free) fraction is the physiologically active, or bioavailable, fraction. Although SHBG binds to other steroid hormones, its affinity for other hormones is lower than that for T, and it accounts for a lower percentage of bound hormone [*e.g.* in women, 45–46% of estradiol (E_2) (4) and 6.63% of androstenedione (5) circulate bound to SHBG]. Thus, an increase in SHBG with estrogen use could lower circulating concentrations of bioavailable T.

T promotes deposition and maintenance of skeletal muscle. Administration of T to elderly men increases the fractional synthetic rate of muscle protein, as well as muscle strength (6). Among normal male subjects, exogenous T increases muscle mass and muscle protein synthesis, and it decreases leucine oxidation (7, 8). By contrast, pharmacologic gonadal suppression in normal young men causes a decrease in whole-body protein synthesis in conjunction with losses of fat-free mass and leg muscle strength (9). Among women with polycystic ovary syndrome, serum T is correlated with lean body mass (10). The decline of T (11–13) and its precursors (14) with age may be one of the factors that contribute to sarcopenia in older men and women.

An age-related loss of muscle mass has been observed in women, and it seems to increase in rate at the time of menopause (15, 16). Loss of muscle mass, particularly in the lower extremity, may lead to loss of physical function, which could increase risk for fall-related injury and other disabilities (17, 18). In addition, the lower total-body energy requirements associated with loss of muscle could increase risk for obesity and associated diseases (15, 19). Thus, factors that accelerate or augment the normal age-related decline in skeletal muscle would be undesirable.

The present cross-sectional study was conducted to determine whether postmenopausal women who used estrogen had higher serum concentrations of SHBG and lower serum concentrations of free T than nonusers, and whether free T was associated with lean body mass, particularly skeletal muscle mass.

Received May 22, 2000. Revision received August 10, 2000. Accepted August 18, 2000.

Address all correspondence and requests for reprints to: Barbara A. Gower, Ph.D., Department of Nutrition Sciences, University of Alabama at Birmingham, 427 Webb Building, 1675 University Boulevard, Birmingham, Alabama 35294-3360. E-mail: bgower@uab.edu.

* Supported by NIA K01-AG-00740 (to B.A.G.) and General Clinical Research Center Grant M01-RR-00032.

Subjects and Methods

Experimental subjects

Subjects were: 70 postmenopausal women, 46–55 yr old (62 Caucasian and 8 African-American). Only women who experienced a natural menopause, with the time of menopause known to occur at least 6 months before contact, were recruited. Both women using hormone replacement therapy (HRT) and women not using HRT were recruited. Among hormone users, only subjects using an oral estrogen preparation were selected (n = 46). Most used conjugated equine estrogens (0.625 mg/day) in combination with medroxyprogesterone acetate (2.5 mg/day), although several women used other types of estrogen or other doses of conjugated equine estrogens. Six women used unopposed estrogen. "No HRT use" (n = 24) was defined as no current use, and no use within the past 6 months. Twenty-one of 24 nonusers had never used HRT. Data were collected over a 27-h period during an in-patient visit to the Department of Nutrition Sciences and the General Clinical Research Center at the University of Alabama at Birmingham (UAB). The protocol was approved by the Institutional Review Board for Human Use at UAB, and all subjects signed an informed consent before testing.

Protocol

Subjects arrived at the Department of Nutrition Sciences, at approximately 0900 h, in the fasted condition (12-h fast). Body composition was determined by dual-energy x-ray absorptiometry (DXA). Three fasting blood samples were taken, over a 40-min period on the following morning, at UAB's General Clinical Research Center at approximately 0700 h. Samples were allowed to clot, then were centrifuged. Sera were aspirated, pooled, aliquoted, and stored at −85 C until used for hormone assay.

Body composition

Total and regional (leg, arm) body composition (fat mass and lean body mass) were measured by DXA using a DPX-L densitometer (Lunar Corp., Madison, WI). Subjects were scanned in light clothing while lying flat on their backs with arms at their sides. DXA scans were performed and analyzed with adult software version 1.5 g. Leg and arm tissue masses were determined by manually placing delimiting lines at specific landmarks on the computer-generated image. The legs were indicated to be all soft tissue below the triangle formed by drawing a horizontal line across the top of the pelvis, and two angled lines (one through each femoral neck). To delineate the arms, lines were drawn between the torso and the arms, from the top of the arm socket to the phalange tips, avoiding contact with the rib and pelvic areas.

Hormone assay

Serum E_2 was measured using a double-antibody RIA (Diagnostic Products, Los Angeles, CA). Sera were first extracted in diethyl ether. Assay sensitivity was determined to be 15.42 pmol/L, intraassay coefficient of variation to be 5.3%, and interassay coefficient of variation to be 6.0%. Serum total T was measured with a coated-tube RIA (Diagnostic Products); assay sensitivity was 0.409 nmol/L, intraassay coefficient of variation was 7.7%, and interassay coefficient of variation was 8.2%. For measurement of SHBG, sera were first diluted 1:101, then assayed in duplicate 25-μL aliquots using an immunoradiometric assay (Diagnostic Systems Laboratories, Inc., Webster, TX); the lower limit was 5 nmol/L, the intraassay coefficient of variation was 8.2%, and the interassay coefficient of variation was 8.2%. Free T (pmol/L) was calculated from serum concentrations of total T and SHBG using the method of Sodergard *et al.* (4). This method is based on the concentration of albumin, the binding capacity of SHBG, and the association constants of T for SHBG and albumin, as determined in a sample of normal men and women.

Statistics

For all analyses, values for body composition variables and serum analytes were log-transformed to produce a normal distribution. ANOVA was used to compare measurements between hormone users and nonusers. Pearson correlation analysis was used to examine associations among serum hormone concentrations and between regional lean mass and serum hormone concentrations. All data were analyzed with SAS for Windows version 6.12. Differences and associations were considered significant if P was less than 0.05.

Results

Descriptive statistics are shown in Table 1. Hormone users and nonusers did not differ, with respect to age, weight, or lean mass (total and regional). Nonusers had a higher total body fat mass and percent body fat. Hormone users had higher serum concentrations of E_2 and SHBG and lower concentrations of free T; total T did not differ (Fig. 1; A, B, C, and D, respectively).

Correlations between lean mass and T (total and free) are shown in Table 2 and Fig. 2. Total lean mass and leg lean mass were significantly correlated with free (but not total) T; arm lean mass was not correlated with either measure of T. Serum E_2 was significantly correlated with SHBG and free T (Fig. 3, A and B, respectively).

Discussion

The present cross-sectional study showed that greater serum SHBG among postmenopausal women using oral estrogen therapy was associated with lower circulating free T. Free T, but not total T, was positively associated with lean

TABLE 1. Descriptive statistics; mean ± SD (range)

	Nonusers (n = 24)	HRT users (n = 46)
Age (yr)	50 ± 3 (46–55)	50 ± 3 (45–57)
Body weight (kg)	73.4 ± 13.3 (54.5–101.7)	67.7 ± 11.2 (47.3–99.3)
Total fat mass (kg)	30.6 ± 9.4 (18.0–51.6)	25.5 ± 8.5 (10.7–42.5)[a]
% Fat	41.5 ± 5.6 (31.5–52.3)	37.3 ± 7.4 (20.4–52.0)[a]
Total lean body mass (kg)	39.3 ± 4.2 (32.9–47.4)	38.9 ± 4.7 (30.3–56.0)
Arm lean mass (kg)	4.3 ± 0.7 (2.9–6.1)	4.3 ± 0.7 (3.0–7.0)
Leg lean mass (kg)	13.6 ± 1.8 (10.9–18.3)	13.3 ± 1.9 (9.4–19.7)
Serum estradiol (pmol/L)	58 ± 69 (15–293)	136 ± 101 (20–616)[b]
Serum SHBG (nmol/L)	82.9 ± 41.1 (23.1–162.8)	182.0 ± 58.5 (92.4–326.2)[b]
Serum total T (nmol/L)	0.68 ± 0.30 (0.41–1.38)	0.67 ± 0.28 (0.41–1.44)
Serum free T (pmol/L)	7.9 ± 4.1 (2.3–17.3)	3.7 ± 2.2 (1.4–12.0)[b]

[a] and [b], $P < 0.05$, 0.001, respectively, *vs.* nonusers.

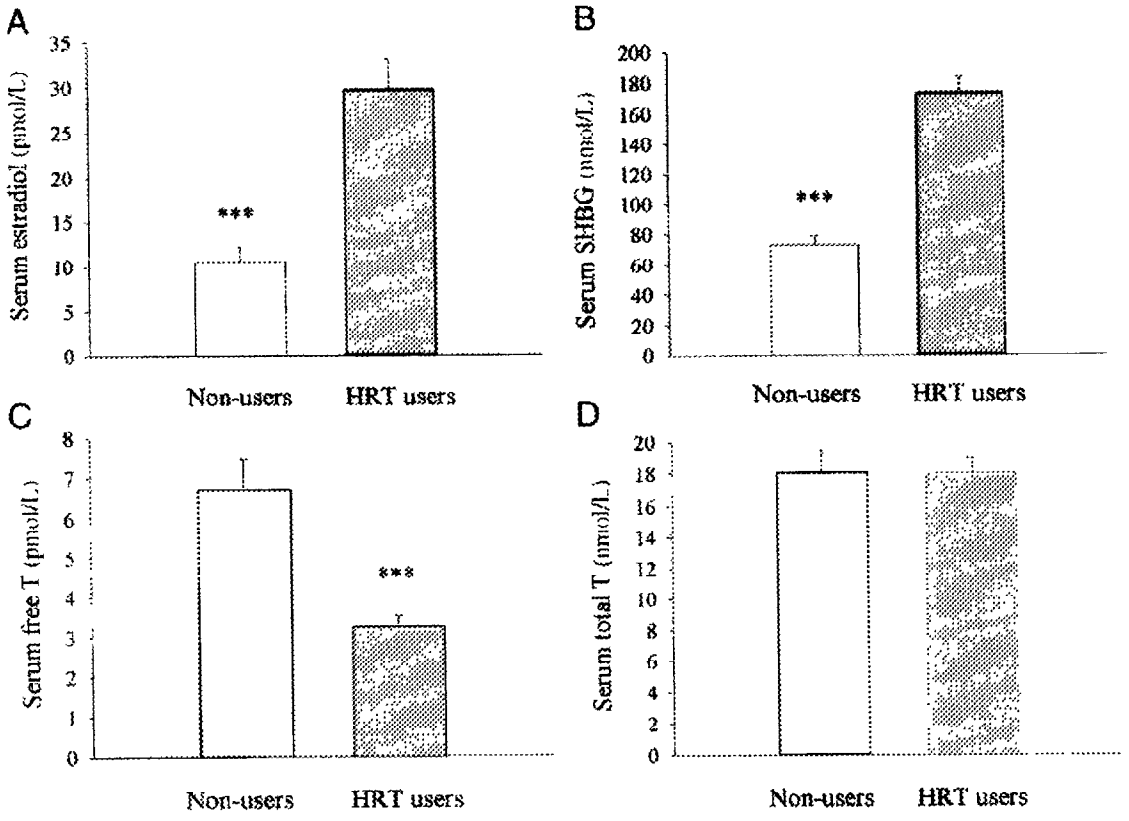

FIG. 1. A, Serum E_2 concentration (pmol/L) in users and nonusers of HRT; ***, $P < 0.01$. B, Serum SHBG concentration (nmol/L) in users and nonusers of HRT; ***, $P < 0.01$. C, Serum free T concentration (pmol/L) in users and nonusers of HRT; ***, $P < 0.01$. D, Serum total T concentration (nmol/L) in users and nonusers of HRT; means were not significantly different.

TABLE 2. Pearson correlation coefficients for serum T concentrations *vs.* lean body mass (all women combined)

	Total T (nmol/L)	Free T (pmol/L)
Total lean mass (kg)	0.10	0.24[a]
Leg lean mass (kg)	0.11	0.31[b]
Arm lean mass (kg)	−0.03	0.14

[a] and [b] $P < 0.05$, 0.01, respectively.

body mass. These observations imply that, by reducing the concentration of bioavailable T, oral estrogen therapy may accelerate or augment lean mass loss among postmenopausal women. This conclusion must be considered tentative, because of the cross-sectional nature of the study design, and awaits confirmation by a longitudinal study.

As reported previously with use of oral contraceptives (20), use of oral estrogen among postmenopausal women in this study was associated with a 2-fold greater concentration of circulating SHBG, relative to women not using hormone therapy. Presumably as a result of this difference in SHBG concentration, the concentration of free T was 50% lower in hormone users *vs.* nonusers. It is generally assumed that only the free fraction of circulating T is biologically active. Unbound T can cross the cell membrane and affect gene transcription. Increased messenger RNA for the anabolic hormone IGF-I and decreased expression of IGF binding protein-4, a protein that inhibits the mitogenic action of IGF-I, were observed in skeletal muscle after T administration (6). Thus, activation of the intramuscular IGF-I system is one mechanism through which T may increases protein synthesis. The possibility that lower free T limits muscle growth or

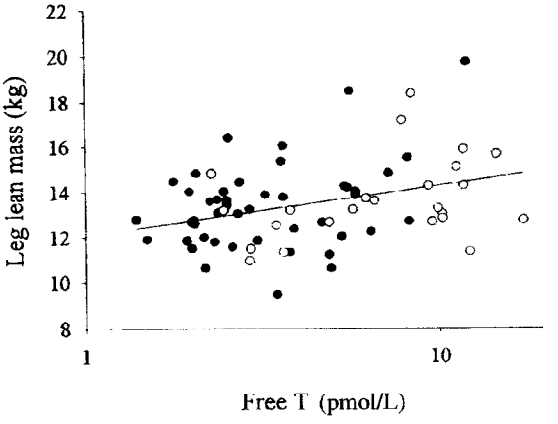

FIG. 2. Leg lean mass (kg) *vs.* serum free T concentration (pmol/L) in all women combined; $P < 0.01$, $R^2 = 0.10$. *Open circles* indicate nonusers and *filled circles* indicate users, respectively, of HRT.

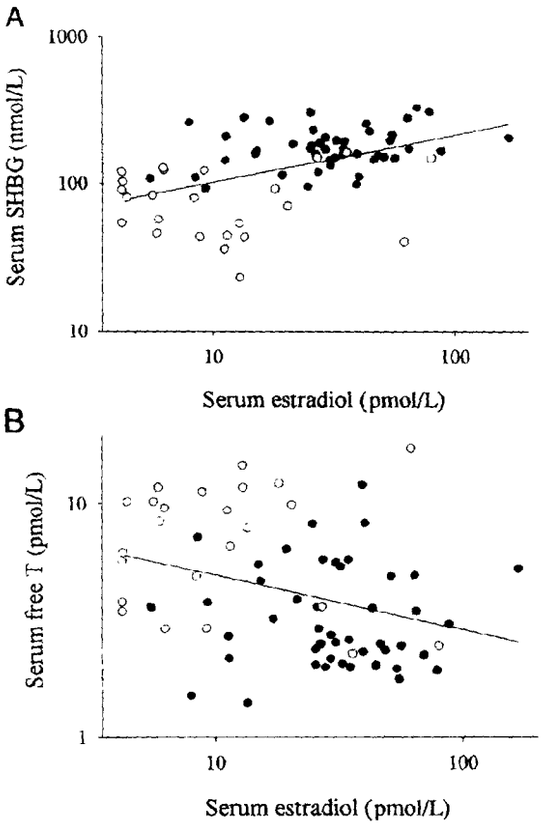

FIG. 3. A, Serum SHBG concentration (nmol/L) *vs.* serum E_2 concentration (pmol/L) in all women combined; $P < 0.001$, $R^2 = 0.24$. B, Serum free T concentration (pmol/L) *vs.* serum E_2 concentration (pmol/L) in all women combined; $P < 0.01$, $R^2 = 0.11$. *Open circles* indicate nonusers and *filled circles* indicate users of HRT, throughout this figure.

maintenance in women using oral estrogen is a topic that has received little attention.

In this study, the concentration of free T was positively correlated with total lean mass and leg lean mass but not arm lean mass. Limb lean mass, as assessed by DXA, is a reasonable surrogate for appendicular skeletal muscle mass (21). The positive association of T with lean mass is logical if T has a positive influence on skeletal muscle accrual or retention. However, in this study, only leg lean mass was associated with serum free T. This association might be explained by the observation that the anabolic effects of T on skeletal muscle are augmented when the muscle is simultaneously stressed by use (22). Thus, it would seem most likely that free T be correlated with those muscles that are used most frequently and experience the greatest loading or force. Whereas leg muscles are used regularly for walking, arm muscles are likely to experience less resistance on a day-to-day basis in individuals who do not engage in resistance training or other activities that involve lifting heavy objects with the arms.

Synthesis of T declines with age in women (12). This decline is marked between the years of 20–30 and 50–60 and is more gradual thereafter (14). A further, transient decline is observed at the time of menopause (23). Thus, at the time of menopause, women are likely to be experiencing a phase of accelerated decline in circulating anabolic hormones. This phenomenon may explain the significant age-independent decline in fat-free mass observed longitudinally in women who traverse the menopause (16).

Many women also initiate use of hormone replacement therapy during the perimenopausal years. The decline in free T associated with oral estrogen use could potentially accelerate the loss of muscle mass that is already occurring because of age- and menopause-related declines in serum T. Loss of muscle mass may be associated with a perceived inability to engage in physical activity, thus resulting in a vicious cycle of inactivity and worsening sarcopenia.

Associations between lean body mass and physical activity have been observed in women. Thirty-nine percent of the decline in fat-free mass with age in women is attributable to a change in maximal oxygen consumption (an index of physical fitness) (15). In addition, leisure time physical activity, as determined by questionnaire, decreases in conjunction with lean body mass during the perimenopausal period (16). Although the cause-and-effect nature of these relationships is not known, it is possible that it could be bidirectional; decreases in muscle mass attributable to hormonal factors could precipitate a decrease in physical activity that, in turn, would lead to further loss of skeletal muscle.

In conclusion, use of oral estrogen therapy was associated with higher SHBG and lower free T among postmenopausal women. Free, but not total, T was associated with total and leg lean body mass. These results imply that use of oral estrogen could accelerate the decline in skeletal muscle mass that occurs with age in women. However, longitudinal observation will be required to determine whether the hypothesis is correct.

Acknowledgments

We acknowledge Kangmei Ren for hormone determinations, Tena Hilario for subject recruitment, Jill Foster and Şola Oguntolu for physician coverage, Stephen J. Winters for the spreadsheet for calculating free T, and the help of the GCRC staff.

References

1. **Helgason S, Damber JE, Damber MG, von Schoultz B, Selstam G, Sodergard R.** 1982 A comparative longitudinal study on sex hormone-binding globulin capacity during estrogen replacement therapy. Acta Obstet Gynecol Scand. 61:97–100.
2. **Campos H, Sacks FM, Walsh BW, Schiff I, O'Hanesian MA, Krauss RM.** 1993 Differential effects of estrogen on low-density lipoprotein subclasses in healthy postmenopausal women. Metabolism 42:1153–1158.
3. **Helle SI, Omsjo IH, Hughes SCC, et al.** 1996 Effects of oral and transdermal oestrogen replacement therapy on plasma levels of insulin-like growth factors and IGF binding proteins 1 and 3: a cross-over study. Clin Endocrinol (Oxf). 45:727–732.
4. **Sodergard R, Backstrom T, Shanbhag V, Carstensen H.** 1982 Calculation of free and bound fractions of testosterone and estradiol-17β to human plasma proteins at body temperature. J Steroid Biochem. 16:801–810
5. **Dunn JF, Nisula BC, Rodbard D.** 1981 Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. J Clin Endocrinol Metab. 53:58–68.
6. **Urban RJ, Bodenburg YH, Gilkison C, et al.** 1995 Testosterone administration to elderly men increases skeletal muscle strength and protein synthesis. Am J Physiol. 269:E820–E826.
7. **Griggs RC, Kingston W, Jozefowicz RF, Herr BE, Forbes G, Halliday D.** 1989 Effect of testosterone on muscle mass and muscle protein synthesis. J Appl Physiol. 66:498–503

8. **Ferrando AA, Tipton KD, Doyle D, Phillips SM, Cortiella J, Wolfe RR.** 1998 Testosterone injection stimulates net protein synthesis but not tissue amino acid transport. Am J Physiol. 275:E864–E871.
9. **Mauras N, Hayes V, Welch S, et al.** 1998 Testosterone deficiency in young men: marked alterations in whole body protein kinetics, strength, and adiposity J Clin Endocrinol Metab. 83:1886–1892.
10. **Douchi T, Yamamoto S, Oki T, Maruta K, Kuwahata R, Nagata Y.** 1999 Serum androgen levels and muscle mass in women with polycystic ovary syndrome. Obstet Gynecol 94:337–340.
11. **Simon D, Preziosi P, Barrett-Connor E, et al.** 1992 The influence of aging on plasma sex hormones in men: the Telecom Study Am J Epidemiol. 135:783–791.
12. **Zumoff B, Strain GW, Miller LK, Rosner W.** 1995 Twenty-four-hour mean plasma testosterone concentration declines with age in normal premenopausal women. J Clin Endocrinol Metab. 80:1429–1430.
13. **Turcato E, Zamboni M, de Pergola G, et al.** 1997 Interrelationships between weight loss, body fat distribution and sex hormones in pre- and postmenopausal obese women. J Intern Med. 241:363–372.
14. **Labrie F, Belanger A, Cusan L, Gomez J-L, Candas B.** 1997 Marked decline in serum concentrations of adrenal C19 sex steroid precursors and conjugated androgen metabolites during aging. J Clin Endocrinol Metab. 82:2396–2402.
15. **Poehlman ET, Goran MI, Gardner AW, et al.** 1993 Determinants of the decline in resting metabolic rate in aging females Am J Physiol. 264:E450–E455.
16. **Poehlman ET, Toth MJ, Gardner AW.** 1995 Changes in energy balance and body composition at menopause: a controlled longitudinal study. Ann Intern Med. 123:673–675.
17. **Guralnik JM, Ferrucci L, Simonsick EM, Salive ME, Wallace RB.** 1995 Lower-extremity function in persons over the age of 70 years as a predictor of subsequent disability. N Engl J Med. 332:556–561.
18. **Wolfson L, Judge J, Whipple R, King M.** 1995 Strength is a major factor in balance, gait, and the occurrence of falls. J Gerontol. 50:64–67.
19. **Tzankoff SP, Norris AH.** 1977 Effect of muscle mass decrease on age-related BMR changes. J Appl Physiol. 43:1001–1006.
20. **Kuhnz W, Staks T, Jütting G.** 1994 Pharmacokinetics of levonorgestrel and ethinylestradiol in 14 women during three months of treatment with a tri-step combination of oral contraceptive: serum protein binding of levonorgestrel and influence on free and total testosterone levels in the serum Contraception. 50:563–579.
21. **Wang Z, Visser M, Ma R, et al.** 1996 Skeletal muscle mass: evaluation of neuron activation and dual-energy x-ray absorptiometry methods. J Appl Physiol. 80:824–831.
22. **Bhasin S, Storer TW, Berman N, et al.** 1996 The effects of supraphysiological doses of testosterone on muscle size and strength in normal men. N Engl J Med. 335:1–7.
23. **Laughlin GA, Barrett-Connor E, Kritz-Silverstein D, von Mühlen D.** 2000 Hysterectomy, oophorectomy, and endogenous sex hormone levels in older women: the Rancho Bernardo Study. J Clin Endocrinol Metab. 85:645–651.

0021-972X/00/$03.00/0
The Journal of Clinical Endocrinology & Metabolism

Vol. 85, No 12
Printed in U.S.A.

Associations among Oral Estrogen Use, Free Testosterone Concentration, and Lean Body Mass among Postmenopausal Women*

BARBARA A. GOWER AND LARA NYMAN

University of Alabama at Birmingham, Department of Nutrition Sciences (B.A.G.), Division of Physiology and Metabolism, and Clinical Nutrition Research Center, and Department of Physiology and Biophysics (L.N.), Birmingham, Alabama 35294-3360

ABSTRACT

Circulating concentrations of sex hormone-binding globulin (SHBG) are increased by use of oral estrogen. The objective of this study was to determine whether postmenopausal women who used oral estrogen had higher serum concentrations of SHBG and lower serum concentrations of free testosterone (T) than nonusers, and whether free T was associated with lean body mass, particularly skeletal muscle mass. Subjects were 70 postmenopausal women, 46–55 yr old, 46 of whom used oral estrogen. Total and regional body composition were determined by dual-energy x-ray absorptiometry. Serum concentrations of SHBG, total T, and estradiol (E_2) were determined by RIA. Free T was calculated from concentrations of total T and SHBG. Hormone users had higher serum concentrations of E_2 and SHBG (182.0 ± 58.5 *vs.* 82.9 ± 41.1 nmol/L, mean ± SD, $P < 0.001$) and lower concentrations of free T (3.7 ± 2.2 *vs.* 7.9 ± 4.1 pmol/L, mean ± SD, $P < 0.001$); total T did not differ. Total lean mass and leg lean mass were significantly correlated with free, but not total T [r values of 0.29 ($P < 0.05$) and 0.31 ($P < 0.01$) for total and leg lean mass, respectively, *vs.* free T]; arm lean mass was not correlated with either measure of T. Serum E_2 was significantly correlated with SHBG ($r = 0.50$, $P < 0.001$) and free T ($r = -0.33$, $P < 0.01$). These observations imply that, by reducing the concentration of bioavailable T, oral estrogen therapy may accelerate or augment lean mass loss among postmenopausal women. This conclusion awaits confirmation by longitudinal observation. (*J Clin Endocrinol Metab* **85**: 4476–4480, 2000)

ORAL ESTROGEN THERAPY, because of the first pass effect, results in exposure of the liver to supraphysiological concentrations of estrogen. One of the consequences of this exposure is an alteration in the production of several hepatic proteins. Concentrations of sex hormone-binding globulin (SHBG) (1) and apolipoprotein A-I (Apo A-I) (2) are higher, and insulin-like growth factor (IGF)-I are lower (3), in women who use oral estrogen therapy, when compared with nonusers.

The physiological ramifications of altered hepatic protein production with estrogen use are not entirely clear. This is particularly true with respect to the elevation in SHBG. SHBG binds to circulating testosterone (T). In premenopausal women, approximately 62% of circulating T is bound to SHBG; another 36–37% is bound to albumin, and approximately 1.5% is unbound (4). It is assumed that the unbound (or free) fraction is the physiologically active, or bioavailable, fraction. Although SHBG binds to other steroid hormones, its affinity for other hormones is lower than that for T, and it accounts for a lower percentage of bound hormone [*e.g.* in women, 45–46% of estradiol (E_2) (4) and 6.63% of androstenedione (5) circulate bound to SHBG]. Thus, an increase in SHBG with estrogen use could lower circulating concentrations of bioavailable T.

T promotes deposition and maintenance of skeletal muscle. Administration of T to elderly men increases the fractional synthetic rate of muscle protein, as well as muscle strength (6). Among normal male subjects, exogenous T increases muscle mass and muscle protein synthesis, and it decreases leucine oxidation (7, 8). By contrast, pharmacologic gonadal suppression in normal young men causes a decrease in whole-body protein synthesis in conjunction with losses of fat-free mass and leg muscle strength (9). Among women with polycystic ovary syndrome, serum T is correlated with lean body mass (10). The decline of T (11–13) and its precursors (14) with age may be one of the factors that contribute to sarcopenia in older men and women.

An age-related loss of muscle mass has been observed in women, and it seems to increase in rate at the time of menopause (15, 16). Loss of muscle mass, particularly in the lower extremity, may lead to loss of physical function, which could increase risk for fall-related injury and other disabilities (17, 18). In addition, the lower total-body energy requirements associated with loss of muscle could increase risk for obesity and associated diseases (15, 19). Thus, factors that accelerate or augment the normal age-related decline in skeletal muscle would be undesirable.

The present cross-sectional study was conducted to determine whether postmenopausal women who used estrogen had higher serum concentrations of SHBG and lower serum concentrations of free T than nonusers, and whether free T was associated with lean body mass, particularly skeletal muscle mass.

Received May 22, 2000. Revision received August 10, 2000. Accepted August 18, 2000.

Address all correspondence and requests for reprints to: Barbara A. Gower, Ph.D., Department of Nutrition Sciences, University of Alabama at Birmingham, 427 Webb Building, 1675 University Boulevard, Birmingham, Alabama 35294-3360. E-mail: bgower@uab.edu.

* Supported by NIA K01-AG-00740 (to B.A.G.) and General Clinical Research Center Grant M01-RR-00032.

Subjects and Methods

Experimental subjects

Subjects were: 70 postmenopausal women, 46–55 yr old (62 Caucasian and 8 African-American). Only women who experienced a natural menopause, with the time of menopause known to occur at least 6 months before contact, were recruited. Both women using hormone replacement therapy (HRT) and women not using HRT were recruited. Among hormone users, only subjects using an oral estrogen preparation were selected (n = 46). Most used conjugated equine estrogens (0.625 mg/day) in combination with medroxyprogesterone acetate (2.5 mg/day), although several women used other types of estrogen or other doses of conjugated equine estrogens. Six women used unopposed estrogen. "No HRT use" (n = 24) was defined as no current use, and no use within the past 6 months. Twenty-one of 24 nonusers had never used HRT. Data were collected over a 27-h period during an in-patient visit to the Department of Nutrition Sciences and the General Clinical Research Center at the University of Alabama at Birmingham (UAB). The protocol was approved by the Institutional Review Board for Human Use at UAB, and all subjects signed an informed consent before testing.

Protocol

Subjects arrived at the Department of Nutrition Sciences, at approximately 0900 h, in the fasted condition (12-h fast). Body composition was determined by dual-energy x-ray absorptiometry (DXA). Three fasting blood samples were taken, over a 40-min period on the following morning, at UAB's General Clinical Research Center at approximately 0700 h. Samples were allowed to clot, then were centrifuged. Sera were aspirated, pooled, aliquoted, and stored at −85 C until used for hormone assay.

Body composition

Total and regional (leg, arm) body composition (fat mass and lean body mass) were measured by DXA using a DPX-L densitometer (Lunar Corp., Madison, WI). Subjects were scanned in light clothing while lying flat on their backs with arms at their sides. DXA scans were performed and analyzed with adult software version 1.5 g. Leg and arm tissue masses were determined by manually placing delimiting lines at specific landmarks on the computer-generated image. The legs were indicated to be all soft tissue below the triangle formed by drawing a horizontal line across the top of the pelvis, and two angled lines (one through each femoral neck). To delineate the arms, lines were drawn between the torso and the arms, from the top of the arm socket to the phalange tips, avoiding contact with the rib and pelvic areas.

Hormone assay

Serum E_2 was measured using a double-antibody RIA (Diagnostic Products, Los Angeles, CA). Sera were first extracted in diethyl ether. Assay sensitivity was determined to be 15.42 pmol/L, intraassay coefficient of variation to be 5.3%, and interassay coefficient of variation to be 6.0%. Serum total T was measured with a coated-tube RIA (Diagnostic Products); assay sensitivity was 0.409 nmol/L, intraassay coefficient of variation was 7.7%, and interassay coefficient of variation was 8.2%. For measurement of SHBG, sera were first diluted 1:101, then assayed in duplicate 25-μL aliquots using an immunoradiometric assay (Diagnostic Systems Laboratories, Inc., Webster, TX); the lower limit was 5 nmol/L, the intraassay coefficient of variation was 8.2%, and the interassay coefficient of variation was 8.2%. Free T (pmol/L) was calculated from serum concentrations of total T and SHBG using the method of Sodergard *et al.* (4). This method is based on the concentration of albumin, the binding capacity of SHBG, and the association constants of T for SHBG and albumin, as determined in a sample of normal men and women.

Statistics

For all analyses, values for body composition variables and serum analytes were log-transformed to produce a normal distribution. ANOVA was used to compare measurements between hormone users and nonusers. Pearson correlation analysis was used to examine associations among serum hormone concentrations and between regional lean mass and serum hormone concentrations. All data were analyzed with SAS for Windows version 6.12. Differences and associations were considered significant if P was less than 0.05.

Results

Descriptive statistics are shown in Table 1. Hormone users and nonusers did not differ, with respect to age, weight, or lean mass (total and regional). Nonusers had a higher total body fat mass and percent body fat. Hormone users had higher serum concentrations of E_2 and SHBG and lower concentrations of free T; total T did not differ (Fig. 1; A, B, C, and D, respectively).

Correlations between lean mass and T (total and free) are shown in Table 2 and Fig. 2. Total lean mass and leg lean mass were significantly correlated with free (but not total) T; arm lean mass was not correlated with either measure of T. Serum E_2 was significantly correlated with SHBG and free T (Fig. 3, A and B, respectively).

Discussion

The present cross-sectional study showed that greater serum SHBG among postmenopausal women using oral estrogen therapy was associated with lower circulating free T. Free T, but not total T, was positively associated with lean

TABLE 1. Descriptive statistics; mean ± SD (range)

	Nonusers (n = 24)	HRT users (n = 46)
Age (yr)	50 ± 3 (46–55)	50 ± 3 (45–57)
Body weight (kg)	73.4 ± 13.3 (54.5–101.7)	67.7 ± 11.2 (47.3–99.3)
Total fat mass (kg)	30.6 ± 9.4 (18.0–51.6)	25.5 ± 8.5 (10.7–42.5)[a]
% Fat	41.5 ± 5.6 (31.5–52.3)	37.3 ± 7.4 (20.4–52.0)[a]
Total lean body mass (kg)	39.3 ± 4.2 (32.9–47.4)	38.9 ± 4.7 (30.3–56.0)
Arm lean mass (kg)	4.3 ± 0.7 (2.9–6.1)	4.3 ± 0.7 (3.0–7.0)
Leg lean mass (kg)	13.6 ± 1.8 (10.9–18.3)	13.3 ± 1.9 (9.4–19.7)
Serum estradiol (pmol/L)	58 ± 69 (15–293)	136 ± 101 (20–616)[b]
Serum SHBG (nmol/L)	82.9 ± 41.1 (23.1–162.8)	182.0 ± 58.5 (92.4–326.2)[b]
Serum total T (nmol/L)	0.68 ± 0.30 (0.41–1.38)	0.67 ± 0.28 (0.41–1.44)
Serum free T (pmol/L)	7.9 ± 4.1 (2.3–17.3)	3.7 ± 2.2 (1.4–12.0)[b]

[a] and [b], $P < 0.05$, 0.001, respectively, *vs.* nonusers.

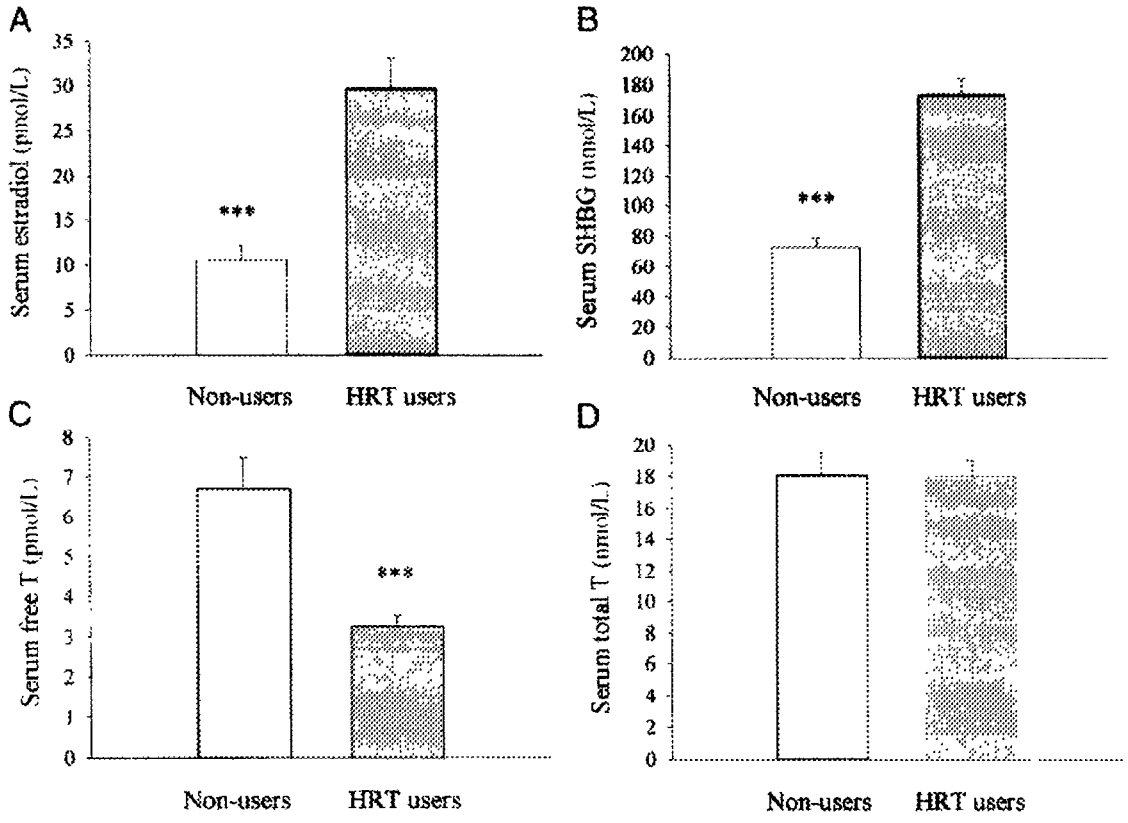

FIG. 1. A, Serum E_2 concentration (pmol/L) in users and nonusers of HRT; ***, $P < 0.01$. B, Serum SHBG concentration (nmol/L) in users and nonusers of HRT; ***, $P < 0.01$. C, Serum free T concentration (pmol/L) in users and nonusers of HRT; ***, $P < 0.01$. D, Serum total T concentration (nmol/L) in users and nonusers of HRT; means were not significantly different.

TABLE 2. Pearson correlation coefficients for serum T concentrations *vs.* lean body mass (all women combined)

	Total T (nmol/L)	Free T (pmol/L)
Total lean mass (kg)	0.10	0.24[a]
Leg lean mass (kg)	0.11	0.31[b]
Arm lean mass (kg)	−0.03	0.14

[a] and [b] $P < 0.05$, 0.01, respectively.

body mass. These observations imply that, by reducing the concentration of bioavailable T, oral estrogen therapy may accelerate or augment lean mass loss among postmenopausal women. This conclusion must be considered tentative, because of the cross-sectional nature of the study design, and awaits confirmation by a longitudinal study.

As reported previously with use of oral contraceptives (20), use of oral estrogen among postmenopausal women in this study was associated with a 2-fold greater concentration of circulating SHBG, relative to women not using hormone therapy. Presumably as a result of this difference in SHBG concentration, the concentration of free T was 50% lower in hormone users *vs.* nonusers. It is generally assumed that only the free fraction of circulating T is biologically active. Unbound T can cross the cell membrane and affect gene transcription. Increased messenger RNA for the anabolic hormone IGF-I and decreased expression of IGF binding protein-4, a protein that inhibits the mitogenic action of IGF-I, were observed in skeletal muscle after T administration (6). Thus, activation of the intramuscular IGF-I system is one mechanism through which T may increases protein synthesis. The possibility that lower free T limits muscle growth or

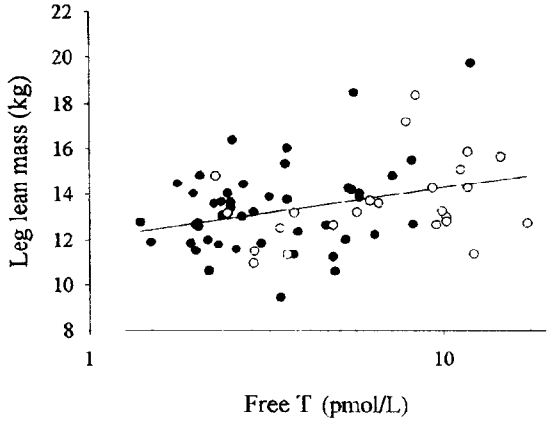

FIG. 2. Leg lean mass (kg) *vs.* serum free T concentration (pmol/L) in all women combined; $P < 0.01$, $R^2 = 0.10$. *Open circles* indicate nonusers and *filled circles* indicate users, respectively, of HRT.

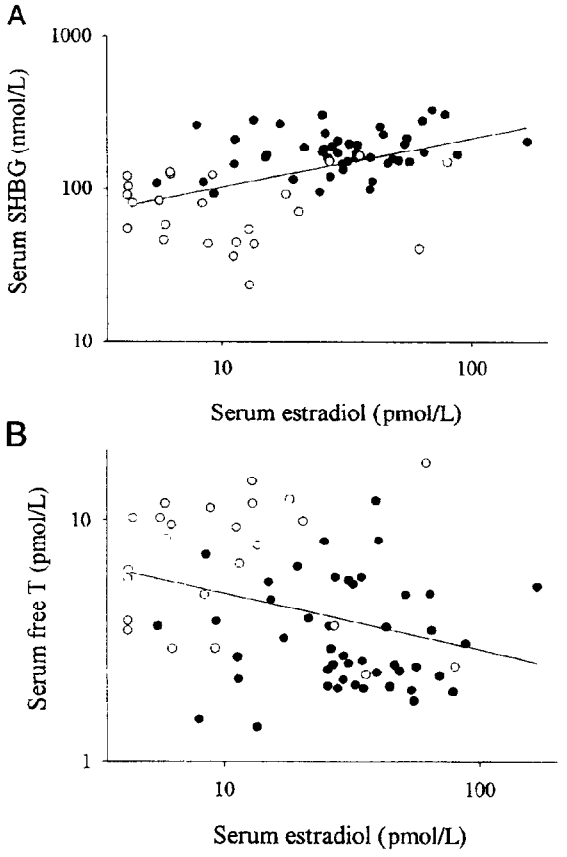

FIG. 3. A, Serum SHBG concentration (nmol/L) *vs.* serum E_2 concentration (pmol/L) in all women combined; $P < 0.001$, $R^2 = 0.24$. B, Serum free T concentration (pmol/L) *vs.* serum E_2 concentration (pmol/L) in all women combined; $P < 0.01$, $R^2 = 0.11$. *Open circles* indicate nonusers and *filled circles* indicate users of HRT, throughout this figure.

maintenance in women using oral estrogen is a topic that has received little attention.

In this study, the concentration of free T was positively correlated with total lean mass and leg lean mass but not arm lean mass. Limb lean mass, as assessed by DXA, is a reasonable surrogate for appendicular skeletal muscle mass (21). The positive association of T with lean mass is logical if T has a positive influence on skeletal muscle accrual or retention. However, in this study, only leg lean mass was associated with serum free T. This association might be explained by the observation that the anabolic effects of T on skeletal muscle are augmented when the muscle is simultaneously stressed by use (22). Thus, it would seem most likely that free T be correlated with those muscles that are used most frequently and experience the greatest loading or force. Whereas leg muscles are used regularly for walking, arm muscles are likely to experience less resistance on a day-to-day basis in individuals who do not engage in resistance training or other activities that involve lifting heavy objects with the arms.

Synthesis of T declines with age in women (12). This decline is marked between the years of 20–30 and 50–60 and is more gradual thereafter (14). A further, transient decline is observed at the time of menopause (23). Thus, at the time of menopause, women are likely to be experiencing a phase of accelerated decline in circulating anabolic hormones. This phenomenon may explain the significant age-independent decline in fat-free mass observed longitudinally in women who traverse the menopause (16).

Many women also initiate use of hormone replacement therapy during the perimenopausal years. The decline in free T associated with oral estrogen use could potentially accelerate the loss of muscle mass that is already occurring because of age- and menopause-related declines in serum T. Loss of muscle mass may be associated with a perceived inability to engage in physical activity, thus resulting in a vicious cycle of inactivity and worsening sarcopenia.

Associations between lean body mass and physical activity have been observed in women. Thirty-nine percent of the decline in fat-free mass with age in women is attributable to a change in maximal oxygen consumption (an index of physical fitness) (15). In addition, leisure time physical activity, as determined by questionnaire, decreases in conjunction with lean body mass during the perimenopausal period (16). Although the cause-and-effect nature of these relationships is not known, it is possible that it could be bidirectional; decreases in muscle mass attributable to hormonal factors could precipitate a decrease in physical activity that, in turn, would lead to further loss of skeletal muscle.

In conclusion, use of oral estrogen therapy was associated with higher SHBG and lower free T among postmenopausal women. Free, but not total, T was associated with total and leg lean body mass. These results imply that use of oral estrogen could accelerate the decline in skeletal muscle mass that occurs with age in women. However, longitudinal observation will be required to determine whether the hypothesis is correct.

Acknowledgments

We acknowledge Kangmei Ren for hormone determinations, Tena Hilario for subject recruitment, Jill Foster and Şola Oguntolu for physician coverage, Stephen J. Winters for the spreadsheet for calculating free T, and the help of the GCRC staff.

References

1. **Helgason S, Damber JE, Damber MG, von Schoultz B, Selstam G, Sodergard R.** 1982 A comparative longitudinal study on sex hormone-binding globulin capacity during estrogen replacement therapy. Acta Obstet Gynecol Scand. 61:97–100.
2. **Campos H, Sacks FM, Walsh BW, Schiff I, O'Hanesian MA, Krauss RM.** 1993 Differential effects of estrogen on low-density lipoprotein subclasses in healthy postmenopausal women. Metabolism 42:1153–1158.
3. **Helle SI, Omsjo IH, Hughes SCC, et al.** 1996 Effects of oral and transdermal oestrogen replacement therapy on plasma levels of insulin-like growth factors and IGF binding proteins 1 and 3: a cross-over study. Clin Endocrinol (Oxf). 45:727–732.
4. **Sodergard R, Backstrom T, Shanbhag V, Carstensen H.** 1982 Calculation of free and bound fractions of testosterone and estradiol-17β to human plasma proteins at body temperature J Steroid Biochem. 16:801–810.
5. **Dunn JF, Nisula BC, Rodbard D.** 1981 Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma J Clin Endocrinol Metab 53:58–68.
6. **Urban RJ, Bodenburg YH, Gilkison C, et al.** 1995 Testosterone administration to elderly men increases skeletal muscle strength and protein synthesis. Am J Physiol. 269:E820–E826.
7. **Griggs RC, Kingston W, Jozefowicz RF, Herr BE, Forbes G, Halliday D.** 1989 Effect of testosterone on muscle mass and muscle protein synthesis. J Appl Physiol 66 498–503.

8. **Ferrando AA, Tipton KD, Doyle D, Phillips SM, Cortiella J, Wolfe RR.** 1998 Testosterone injection stimulates net protein synthesis but not tissue amino acid transport. Am J Physiol. 275:E864–E871
9. **Mauras N, Hayes V, Welch S, et al.** 1998 Testosterone deficiency in young men: marked alterations in whole body protein kinetics, strength, and adiposity J Clin Endocrinol Metab. 83:1886–1892.
10. **Douchi T, Yamamoto S, Oki T, Maruta K, Kuwahata R, Nagata Y.** 1999 Serum androgen levels and muscle mass in women with polycystic ovary syndrome. Obstet Gynecol 94:337–340.
11. **Simon D, Preziosi P, Barrett-Connor E, et al.** 1992 The influence of aging on plasma sex hormones in men: the Telecom Study. Am J Epidemiol. 135:783–791.
12. **Zumoff B, Strain GW, Miller LK, Rosner W.** 1995 Twenty-four-hour mean plasma testosterone concentration declines with age in normal premenopausal women. J Clin Endocrinol Metab. 80:1429–1430.
13 **Turcato E, Zamboni M, de Pergola G, et al.** 1997 Interrelationships between weight loss, body fat distribution and sex hormones in pre- and postmenopausal obese women. J Intern Med. 241:363–372.
14 **Labrie F, Belanger A, Cusan L, Gomez J-L, Candas B.** 1997 Marked decline in serum concentrations of adrenal C19 sex steroid precursors and conjugated androgen metabolites during aging. J Clin Endocrinol Metab. 82:2396–2402.
15. **Poehlman ET, Goran MI, Gardner AW, et al.** 1993 Determinants of the decline in resting metabolic rate in aging females Am J Physiol. 264:E450–E455.
16. **Poehlman ET, Toth MJ, Gardner AW.** 1995 Changes in energy balance and body composition at menopause: a controlled longitudinal study. Ann Intern Med. 123:673–675.
17. **Guralnik JM, Ferrucci L, Simonsick EM, Salive ME, Wallace RB.** 1995 Lower-extremity function in persons over the age of 70 years as a predictor of subsequent disability. N Engl J Med. 332:556–561.
18. **Wolfson L, Judge J, Whipple R, King M.** 1995 Strength is a major factor in balance, gait, and the occurrence of falls. J Gerontol. 50:64–67.
19 **Tzankoff SP, Norris AH.** 1977 Effect of muscle mass decrease on age-related BMR changes. J Appl Physiol. 43:1001–1006.
20 **Kuhnz W, Staks T, Jütting G.** 1994 Pharmacokinetics of levonorgestrel and ethinylestradiol in 14 women during three months of treatment with a tri-step combination of oral contraceptive: serum protein binding of levonorgestrel and influence on free and total testosterone levels in the serum. Contraception. 50:563–579.
21. **Wang Z, Visser M, Ma R, et al.** 1996 Skeletal muscle mass: evaluation of neuron activation and dual-energy x-ray absorptiometry methods. J Appl Physiol 80:824–831.
22. **Bhasin S, Storer TW, Berman N, et al.** 1996 The effects of supraphysiological doses of testosterone on muscle size and strength in normal men. N Engl J Med. 335:1–7.
23 **Laughlin GA, Barrett-Connor E, Kritz-Silverstein D, von Mühlen D.** 2000 Hysterectomy, oophorectomy, and endogenous sex hormone levels in older women: the Rancho Bernardo Study. J Clin Endocrinol Metab 85:645–651.

Volume 58, Number 7
OBSTETRICAL AND GYNECOLOGICAL SURVEY

CME REVIEW ARTICLE 20

CHIEF EDITOR'S NOTE: This article is part of a series of continuing education activities in this Journal through which a total of 36 AMA/PRA category 1 credit hours can be earned in 2003. Instructions for how CME credits can be earned appear on the last page of the Table of Contents.

Transdermal Testosterone for Women: A New Physiological Approach for Androgen Therapy

Norman A. Mazer, MD, PhD*, and Jan L. Shifren, MD†

*Senior Medical Research Fellow, Department of Medical Affairs, Watson Laboratories, Inc. and Adjunct Professor, Department of Pharmaceutics, University of Utah, Salt Lake City, Utah; and †Director of the Menopause Program, Vincent Memorial Obstetrics and Gynecology Service, Massachusetts General Hospital, and Assistant Professor of Obstetrics, Gynecology and Reproductive Biology, Harvard Medical School, Boston, MA

Transdermal testosterone patches and topically applied gels have become well accepted for the treatment of testosterone deficiency in men and are currently being developed in appropriate dosage strengths for androgen therapy in women. The furthest developed among these products is an investigational testosterone matrix patch which is now in phase III clinical trials for the treatment of sexual dysfunction in oophorectomized and naturally menopausal women. This review article discusses the biopharmaceutical rationale for the transdermal delivery of testosterone to women, illustrates and quantitatively analyzes the pharmacokinetics and metabolism of the testosterone matrix patch and a recently investigated testosterone gel, and summarizes the efficacy and safety data that have been reported in phase II studies of the testosterone matrix patch in surgically menopausal women with sexual dysfunction and HIV-infected women with the AIDS wasting syndrome. The different effects of oral and transdermal estrogen therapy (ET) on the concentrations of total and free testosterone attained with the testosterone matrix patch are contrasted. Although still in development, transdermal testosterone therapy appears to be a promising new approach for providing physiologically based androgen therapy to women.

Target Audience: Obstetricians & Gynecologists, Family Physicians

Learning Objectives: After completion of this article, the reader will be able to list the biopharmaceutical rationale for developing the testosterone transdermal delivery system, to describe the pharmacokinetics and metabolism of testosterone transdermal delivery systems in women, and to illustrate the influence of concomitant estrogen therapy on testosterone concentrations attained with the transdermal delivery system.

INTRODUCTION

In contrast to the accepted physiological roles of testosterone in the male and the importance of diagnosing and treating testosterone deficiency in men, the physiological roles of testosterone in women are only beginning to be understood; approaches for diagnosing and treating female androgen insufficiency are at an early stage (1-4). Transdermal testosterone patches and gel products developed for daily application have become well accepted therapies for hypogonadal men in the past decade (5-7) but are contraindicated for use in women because of their high dosages. Nevertheless, the transdermal route is well suited for administering testosterone to women, particularly at the lower dosage rates re-

Reprint requests to: Dr. Norm Mazer, Watson Laboratories, Inc., 417 Wakara Way Suite 100, Salt Lake City, UT 84108.

Dr. Mazer has disclosed that he is an employee of Watson Laboratories, Inc.

Dr. Shifren has disclosed that she receives grant/research support from Procter & Gamble Pharmaceuticals and Watson Laboratories; and is a member of the scientific advisory board of Watson Laboratories.

The authors disclose that the testosterone matrix patch has not been approved by the U.S. Food and Drug Administration.

quired to produce levels of testosterone and its metabolites within the normal female ranges. These rates are based upon the 300 μg per day of testosterone produced daily in healthy young women, about one-twentieth of the daily testosterone production rate in men (1). From a pharmaceutical perspective, the lower testosterone dosage requirement for women enables the development of smaller, thinner and better-tolerated transdermal matrix patches, that can be worn for multiple days (8, 9). To this end, a number of recently reported phase I and phase II studies have evaluated the pharmacokinetics, efficacy and safety of an alcohol-free transdermal testosterone matrix patch applied twice weekly to the abdomen (10-13). The testosterone matrix patch for women is currently being investigated in multi-center phase III trials for the treatment of sexual dysfunction in oophorectomized and naturally postmenopausal women, and in smaller trials for the treatment of HIV-infected women with the AIDS wasting syndrome (14-16). Early efforts to develop a transdermal testosterone gel for women have also been reported (17).

This article will review the current status and development of transdermal testosterone delivery systems for women. We first discuss the biopharmaceutical rationale for transdermal testosterone delivery to women. The pharmacokinetic profiles of testosterone and its metabolites from studies of the testosterone matrix patch and transdermal testosterone gel in surgically menopausal women are then shown and interpreted. The potential impact of concomitant estrogen therapies, which can modify the levels of sex hormone-binding globulin (SHBG) and thereby alter the binding and metabolic clearance of testosterone, on the pharmacokinetics of testosterone are illustrated with data from women concomitantly treated with the testosterone matrix patch and either transdermal estradiol or conjugated equine estrogens. Lastly, we review the efficacy and safety data from published phase II studies of the testosterone matrix patch in surgically menopausal women and in HIV-infected women.

For information on the endocrine control of testosterone production in women, the pathophysiologic states in which it is reduced, and a consensus report on the definition, classification and assessment of female androgen insufficiency, the interested reader is referred to 2 recent review articles (1, 2).

BIOPHARMACEUTICAL RATIONALE FOR TRANSDERMAL TESTOSTERONE DELIVERY TO WOMEN

As in the case of men, the transdermal administration of testosterone to women has a compelling biopharmaceutical rationale, which is explained using the schematic diagram of transdermal permeation, metabolism and absorption (Fig. 1) (18). First, the short serum half-life of testosterone, e.g., ~70 minutes in men (and possibly longer in women) necessitates a sustained input of hormone to maintain serum testosterone levels in the desired physiological or therapeutic range. This can be provided by a transdermal patch that delivers testosterone continuously to the skin by passive diffusion or by using a topical gel, which produces a drug depot within the outer layers of the skin. Second, as a lipophilic steroid hormone, testosterone can readily permeate the stratum corneum barrier of the skin, transit the epidermis, and reach the dermal capillaries where it is absorbed. Third, as the 5∝-reductase and aromatase activities present in the epidermal layer of nongenital skin (such as the abdomen) are relatively small, the degree of first-pass dermal metabolism of testosterone to dihydrotestosterone (DHT) and estradiol (E_2) is likewise expected to be small when using a nongenital transdermal patch (19, 20). The levels of DHT and E_2 attained with the patch should, therefore, largely reflect the systemic conversion of testosterone to DHT and E_2 by the liver, skin and other target organs, e.g., adipose tissue and brain (21, 22). It is noteworthy that a testosterone patch developed for trans-scrotal application in men produces supraphysiological ratios of DHT/T related to the relatively high levels of 5-∝ reductase activity in scrotal skin, whereas the E_2/T ratios are normal, indicating lower levels of aromatase (23). A quantitative analysis of the dermal first-pass effect in scrotal skin suggested that 16-20% of the testosterone was metabolized to DHT before absorption (24).

An important difference in the transdermal delivery of testosterone to women as compared to men is the markedly smaller delivery rate required, i.e., 150-300 μg/day for women versus 2.5-10 mg/day for men, a difference of about 20-fold (1). As a consequence of the smaller dosing requirement, transdermal testosterone delivery to women can be accomplished by using an alcohol-free matrix patch that is worn for multiple days, similar to the second generation of transdermal estradiol patches (8, 9, 25). In contrast to the testosterone reservoir patches or scrotal patch that are applied daily by men, the matrix

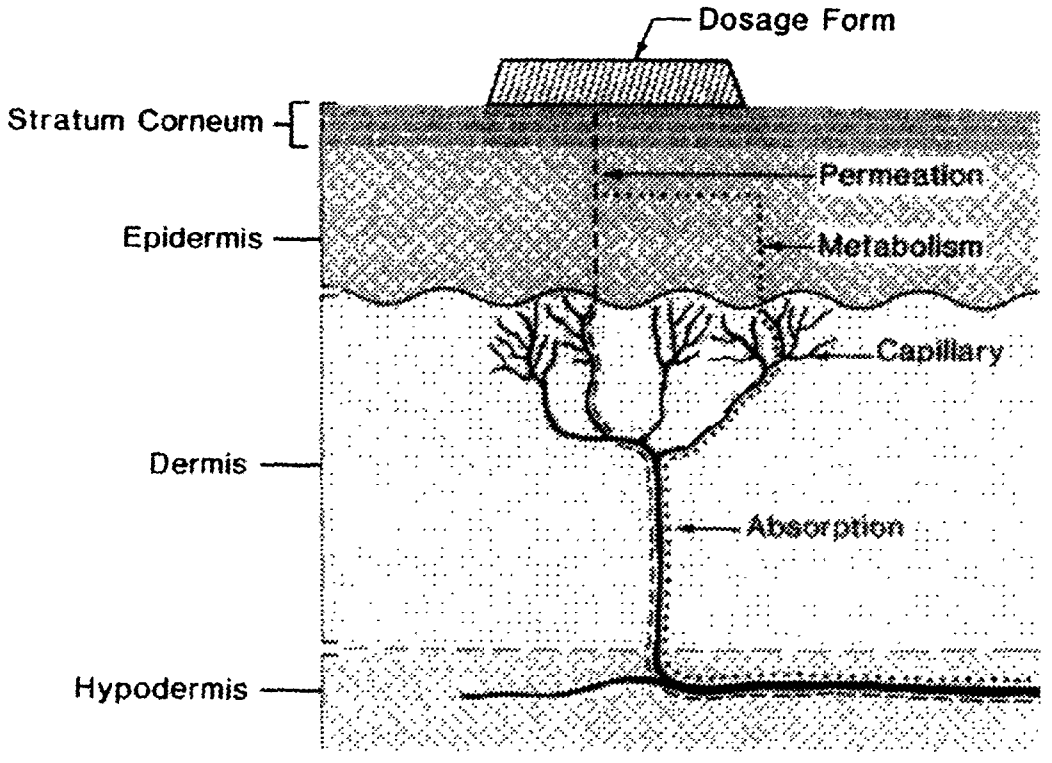

Figure 1. Schematic diagram of transdermal drug transport, including the possibility of transdermal "first-pass" metabolism. Reprinted with permission from Mazer (18).

patches for women are smaller, more cosmetically acceptable and generally much better tolerated locally.

A final consideration in the biopharmaceutical rationale for transdermal testosterone delivery in women is the absence or reduction of hepatic first-pass effects that occur with the oral administration of androgens. These effects include marked reductions in the concentrations of SHBG and thyroxine-binding globulin (26, 27), which may impact hormone bioavailability, and HDL cholesterol (28), which may adversely affect cardiovascular risk.

PHARMACOKINETICS AND METABOLISM OF TESTOSTERONE TRANSDERMAL DELIVERY SYSTEMS IN WOMEN

Experimental Testosterone Transdermal Matrix Patch in Surgically Menopausal Women

Experimental testosterone transdermal patches are currently being developed to provide physiological testosterone treatment for women (29). The testosterone patches for women are alcohol-free matrix patches that are applied to the abdomen twice a week. They have been designed to deliver testosterone at rates of 150 and 300 μg/day, corresponding approximately to 50% and 100% of the daily testosterone production rate in premenopausal women.

The pharmacokinetics of total and free testosterone concentrations before, during and after a 4-day application of the 150 μg/day patch were evaluated in 11 oophorectomized women who had been withdrawn from prior estrogen therapy (Fig. 2 A, B) (1, 10, 29). Before patch application, the baseline testosterone levels were subnormal and exhibited a modest circadian pattern, consistent with the adrenal rhythm of testosterone production (1). After patch application, the levels rose to the mid- and upper-normal ranges and remained relatively constant for 96 hours. Upon patch removal, the levels returned to the baseline pattern within 12-24 hours. The mean time-averaged increments in the total and free testosterone concentrations (above baseline) were 27.6 ± 13.1 (SD) ng/dl and 2.7 ± 0.9 pg/ml, respectively. Assuming a consensus literature value for the metabolic clearance rate of testosterone in women, i.e., 600 liters/day (30-33), the increment in total testosterone corresponds to a mean systemic delivery rate

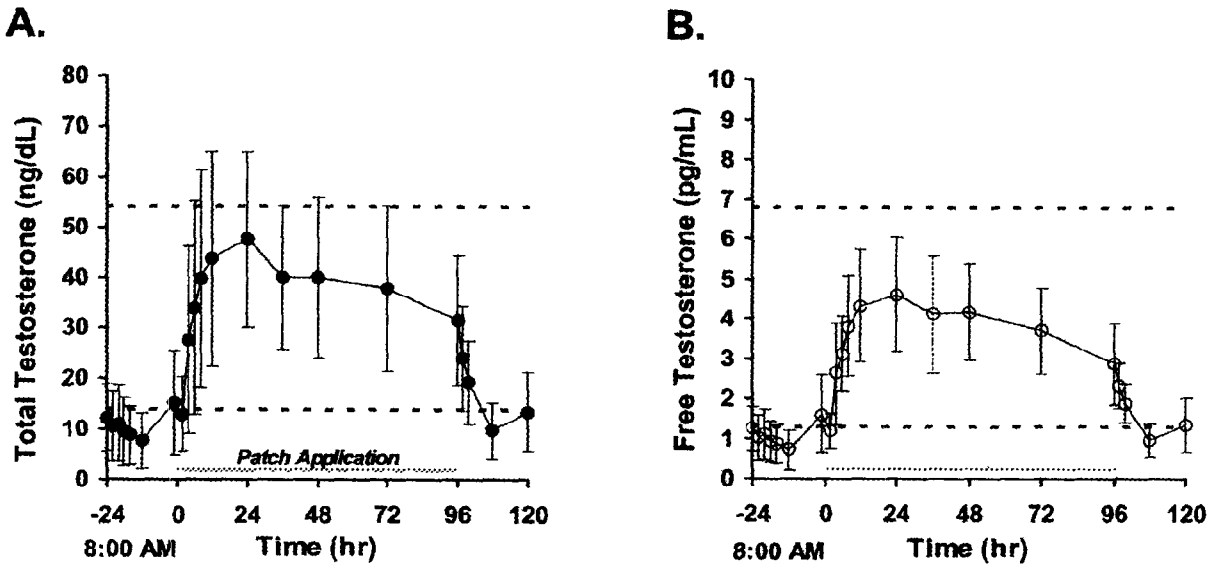

Figure 2 A and B. Mean (± SD) of A, total testosterone, and B, free testosterone concentration profiles measured in 11 oophorectomized women before, during and after a 96-hour application of an experimental testosterone transdermal matrix patch designed to deliver testosterone at a rate of 150 μg/day. All subjects had been withdrawn from estrogen therapy for 4 weeks. Dashed lines denote normal ranges in premenopausal women. Adapted with permission from references 1 and 29.

of 166 μg/day. DHT concentrations showed a profile similar to that of testosterone, attaining stable concentrations in the mid-normal range, whereas estradiol concentrations showed no detectable change during the patch application and remained subnormal (Fig. 3A and B). Analysis of the baseline-subtracted hormone concentrations yielded a mean DHT/T ratio of 0.152 ± 0.042 and a mean E_2/T ratio of 0.004 ±

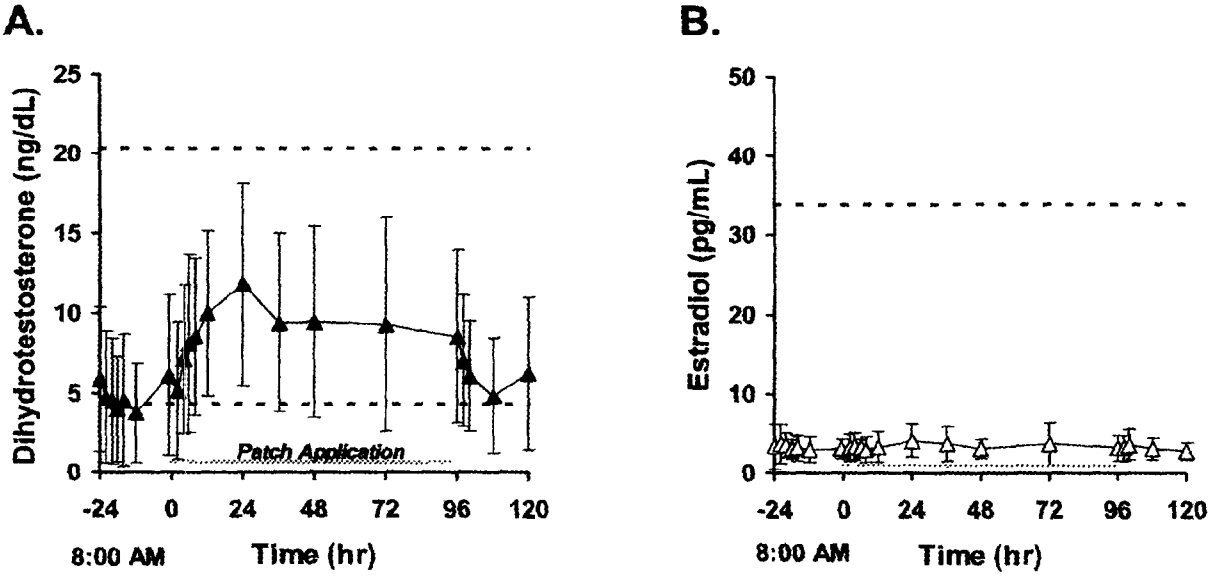

Figure 3 A and B. Mean (± SD) of A, dihydrotestosterone, and B, estradiol concentration profiles measured in 11 oophorectomized women before, during and after a 96-hour application of an experimental testosterone transdermal matrix patch designed to deliver testosterone at a rate of 150 μg/day. All subjects had been withdrawn from estrogen therapy for 4 weeks. Dashed lines denote normal ranges in premenopausal women. Data from AJ Bowen et al (10).

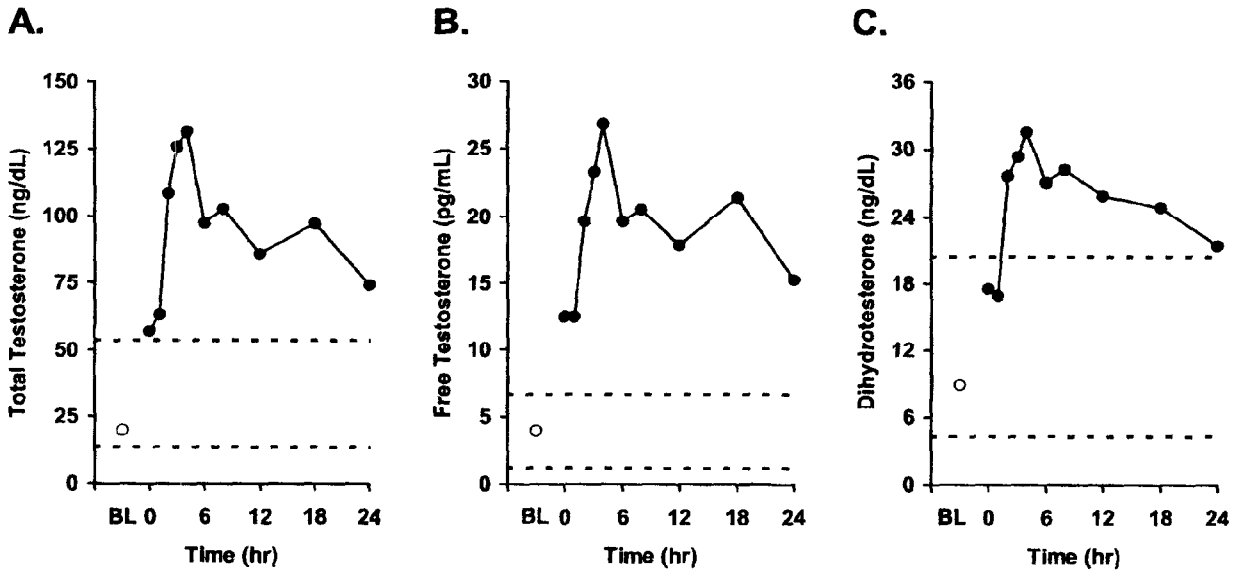

Figure 4 A, B and C. Mean values (filled circles) of A, total testosterone, B, free testosterone, and C, dihydrotestosterone concentration profiles measured in 5 oophorectomized women after 14 consecutive morning applications of a topical gel containing 1 mg of micronized testosterone. White circles denote baseline (BL) values. All subjects were using some form of estrogen therapy. Data from Slater et al (17). Dashed lines denote normal ranges in premenopausal women from Shifren et al (13).

0.006. The DHT/T ratio is significantly greater than the reported systemic conversion ratio of T to DHT in women, i.e., 0.029 (21), suggesting that a small degree of dermal first-pass conversion of T to DHT may be occurring. Based on the model used to interpret the DHT/T ratio of the trans-scrotal patches (24), the present data are consistent with a dermal conversion of T to DHT of approximately 6%. On the other hand, the observed E_2/T ratio (and its variance) is not statistically different from the very small conversion ratio of T to E_2 reported in women, i.e., 0.0005 (22), suggesting that there is no detectable dermal conversion of T to E_2. It also confirms that the systemic conversion of T to E_2 has a negligible effect on circulating E_2 concentrations in women (22). In connection with our later discussion on the effects of estrogen therapy on testosterone levels, it should be noted that the patients in this study had not received oral or transdermal ET for at least 6 weeks, and that their SHBG levels averaged 52.7 ± 29.7 nmol/liter, well within the normal range for premenopausal women (13).

Experimental Testosterone Transdermal Matrix Patch in Healthy and HIV-Infected Women

Additional pharmacokinetic studies have evaluated the testosterone matrix patch in healthy premenopausal women and in HIV-infected women, with both groups applying two patches simultaneously for a total daily dose of 300 μg/day (11). In the healthy women, the mean time-averaged increments in total and free testosterone were 34.6 ng/dl and 5.2 pg/ml, respectively, approximately consistent with a doubling of the free testosterone increment obtained in the surgically menopausal women when given at half the dose (150 μg/day). For the HIV-infected women, the corresponding values were 15.7 ng/dl and 3.0 pg/ml, respectively, suggesting either decreased absorption from the patches or increased metabolic clearance of testosterone. Javanbakht et al.(11) suggest that the latter is more likely responsible for their findings, based on the particular HIV medications used by their patients, which are known to induce hepatic P-450 enzymes that are responsible for testosterone metabolism. However, in an earlier phase II study of HIV-infected women with AIDS wasting syndrome, Miller et al.(12) found that the same testosterone matrix patches produced substantially higher increments of total and free testosterone than seen in surgically menopausal women. The different HIV medications used by Miller's patients and greater degree of wasting may account for the disparate findings between the two studies of HIV-infected women.

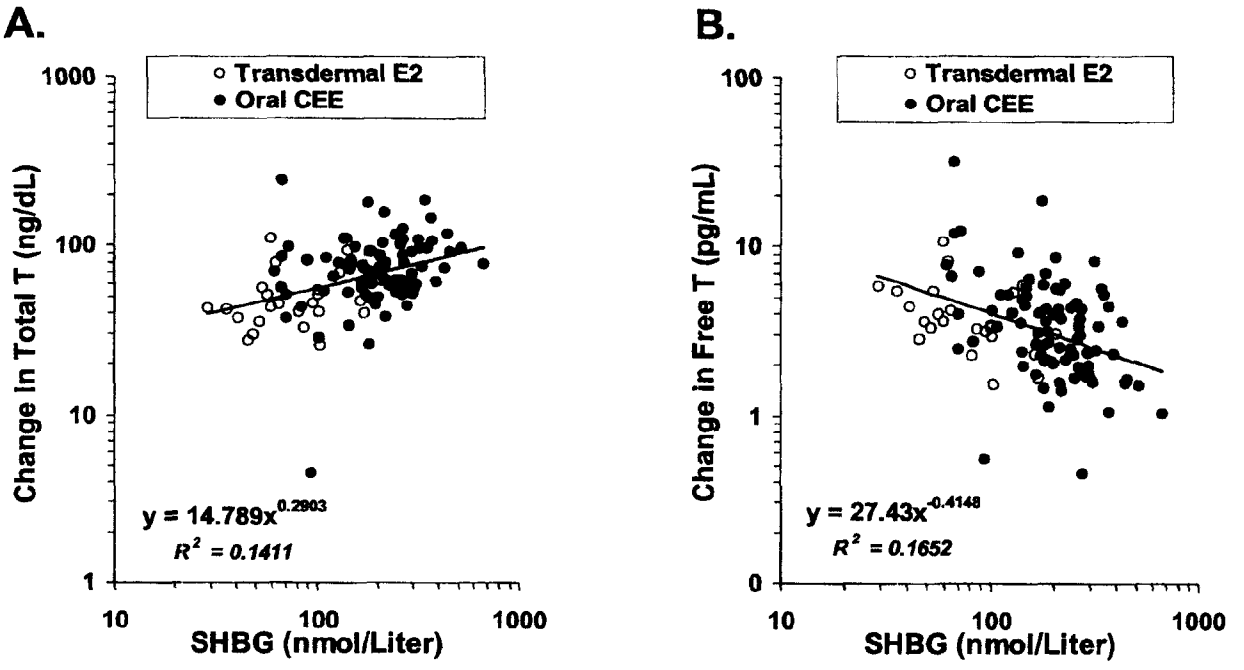

Figure 5 A and B. Individual changes from baseline concentrations of A, total testosterone, and B, free testosterone plotted vs. the baseline SHBG levels in surgically menopausal women treated with the testosterone matrix transdermal patch (300 μg/day delivery rate). White circles denote women concomitantly treated with transdermal estradiol (n = 24). Black circles denote women concomitantly treated with conjugated equine estrogens (n = 89). The changes in free or total testosterone concentrations correspond to the time-average increments measured in steady-state pharmacokinetic studies or the average change from baseline in morning samples obtained during three months of chronic treatment. Equations in the lower left of each figure correspond to a "Power Law" regression analysis of the data from all subjects (n = 113), represented by the straight lines. Adapted from Rosario-Janssen and Mazer (37).

Topical Testosterone Containing Gel in Surgically Menopausal Women

Topical gels containing micronized testosterone are compounded and sold in specialized compounding pharmacies in the U.S. One of the few studies to evaluate the pharmacokinetics and metabolism of such a product was recently reported by Slater et al. (17). The mean concentration profiles of total testosterone, free testosterone and DHT, corresponding to 14 days of daily morning application to the inner thigh of a topical gel containing 1 mg of micronized testosterone, was measured in 5 oophorectomized women taking estrogen therapy (Fig. 4 A,B,C). The profiles exhibit an initial peak over the first 4 hours followed by a slow decline over the remaining 24-hour dosing interval. The time-averaged increments of total testosterone (74.5 ng/dl), free testosterone (15.6 pg/ml) and DHT (16.0 ng/dl) resulted in concentrations that substantially exceeded the upper limits of the normal range for premenopausal women (as given in reference 13). Assuming a metabolic clearance rate of 600 liters/day, the increment in total testosterone corresponds to a daily input of approximately 450 μg, nearly 50% of the applied dose. The DHT/T ratio (calculated from the mean time-averaged increments of each hormone) was 0.215, about 40% greater than the value for the testosterone matrix patch. The larger ratio may indicate a somewhat greater degree of dermal first-pass metabolism with the topical application, which could be related to the longer residence time of testosterone in the skin. Estrogen concentrations were reported not to increase during the testosterone gel application, similar to the findings with the testosterone matrix patch. Although the authors of this study felt that the hormone and metabolite profiles achieved with the testosterone gel had a desirable shape, they concluded that the 1-mg dose administered might be excessive.

INFLUENCE OF CONCOMITANT ESTROGEN THERAPY ON THE TESTOSTERONE CONCENTRATIONS ATTAINED WITH THE TRANSDERMAL MATRIX PATCH

Treatment with oral estrogens increases the serum concentrations of SHBG and other hormone-binding proteins made in the liver in a dose-dependent manner (34). In contrast, transdermal estradiol does not exert this hepatic "first-pass" effect. In accordance with the law of mass action, elevations in SHBG concentration increase the amount of testosterone that is bound to SHBG. In turn, this lowers both the metabolic clearance rate of testosterone as well as the free testosterone fraction (35). At a constant level of testosterone production or a constant transdermal delivery rate, the lower metabolic clearance rate leads to an increase in the total testosterone concentration. Because the free testosterone concentration is the product of the total testosterone concentration and the free fraction, the effect of elevated SHBG levels on free testosterone concentrations depends on whether the free fraction decreases more or less strongly than the metabolic clearance rate as the SHBG concentration increases (36). Experimental data presented below indicate that the free fraction decreases more strongly than the metabolic clearance rate, and therefore the free testosterone concentration decreases as the SHBG concentration increases.

Because oral and transdermal estrogen have different effects on SHBG levels, the concentrations of total and free testosterone attained with the matrix patch are expected to differ, depending on the type of concomitant estrogen therapy. These relationships are illustrated by plotting the individual increments in total and free testosterone concentrations resulting from concomitant treatment with the testosterone matrix patch (300 μg/day dose) and different regimens of ET versus the individual SHBG concentrations (Fig. 5 A and B). The data were obtained from a combined population of surgically menopausal women who had been on stable regimens of either transdermal estradiol (0.05 or 0.1 mg/day; n = 24) or oral conjugated equine estrogens (CEE) (0.625 mg/day or greater; n = 89) (37). Although the subjects on CEE generally had higher SHBG levels (median 206 nmol/liter) compared to those on transdermal estradiol (73.2 nmol/liter), there was considerable overlap between these sub-populations. Regardless of the type of ET, the individual data taken as a whole were well described by "power law" regression lines, the equations and r values of which are shown in the plots[a]. For the total testosterone increments, the power law had a positive exponent of 0.2903, whereas for free testosterone increments, the power law had a negative exponent of −0.4148 ($P < 10^{-4}$ for total testosterone and $P < 10^{-5}$ for free testosterone).

Although other factors besides SHBG level clearly contribute to the variability of the total and free testosterone increments in the population analyzed, the influence of ET is clearly relevant to the individual patient, whose SHBG level could vary markedly by the type of ET. As an illustration, using the median SHBG values given above for transdermal and oral ET, the calculated increments in total testosterone for a 300 μg/day testosterone matrix patch would be 51.4 ng/dl with transdermal E_2 versus 69.4 ng/dl for oral CEE. Similarly, the calculated increments in free testosterone would be 4.6 pg/ml for transdermal E_2 versus 3.0 pg/ml for oral CEE. These calculations indicate that for the individual patient who requires both testosterone and ET, the use of transdermal E_2 would result in a substantially greater increase in free testosterone levels than if oral estrogen therapy were given concomitantly with a testosterone matrix patch.

EFFICACY AND SAFETY OF TRANSDERMAL TESTOSTERONE ADMINISTRATION IN WOMEN

Treatment of Sexual Dysfunction in Oophorectomized Women

Shifren et al. (13) reported on a phase II study of the experimental testosterone matrix patches in a population of 75 women with impaired sexual function after oophorectomy. The women (ages 31 to 56 years) received oral CEEs (0.625 mg/day or greater) and in random order, 0 (placebo), 150 μg/day and 300 μg/day testosterone, each for 12-week periods in a double-blind crossover design.

Free testosterone concentrations, which were subnormal at baseline and during placebo, increased to the mid-normal range with 150 μg/day treatment and to the upper normal range with 300 μg/day treatment (Fig. 6A). Total testosterone concentrations increased with dose into the supra-physiological range (Fig. 6B), a consequence of the supra-physiological

[a]Power law regression is used to describe relationships between variables that appear linear when plotted on log-log graphs. The exponents in the power law equations are identical to the slopes of the regression lines shown in such plots.

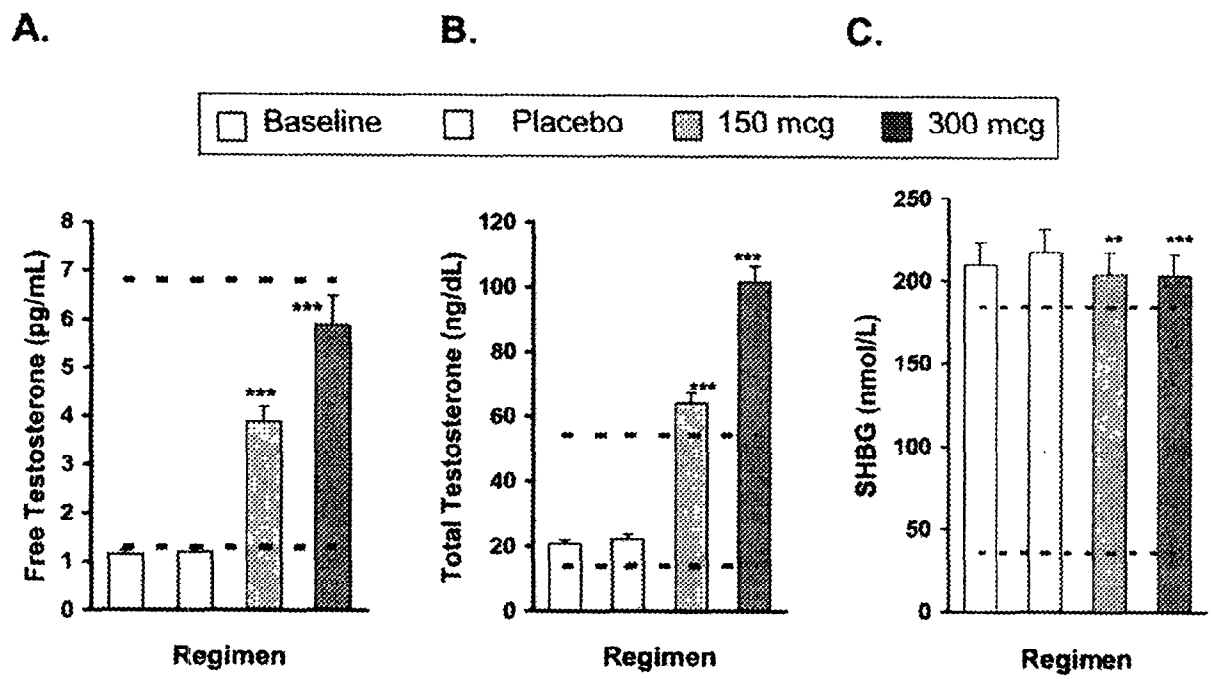

Figure 6. Mean (± SEM) hormone levels measured at baseline and during treatment with experimental transdermal testosterone patches at doses of 0 (placebo), 150 and 300 μg/day from study of 75 oophorectomized with impaired sexual function. (A) Free testosterone levels. (B) Total testosterone levels. (C) Sex hormone-binding (SHBG) levels. All patients received oral CEEs at doses of 0.625 mg/day or greater. Dashed lines represent normal reference ranges in healthy premenopausal women. **, $P < 0.01$; ***, $P < 0.001$ vs. placebo. Data from Shifren et al (13). Panel A is reprinted with permission from Mazer (1).

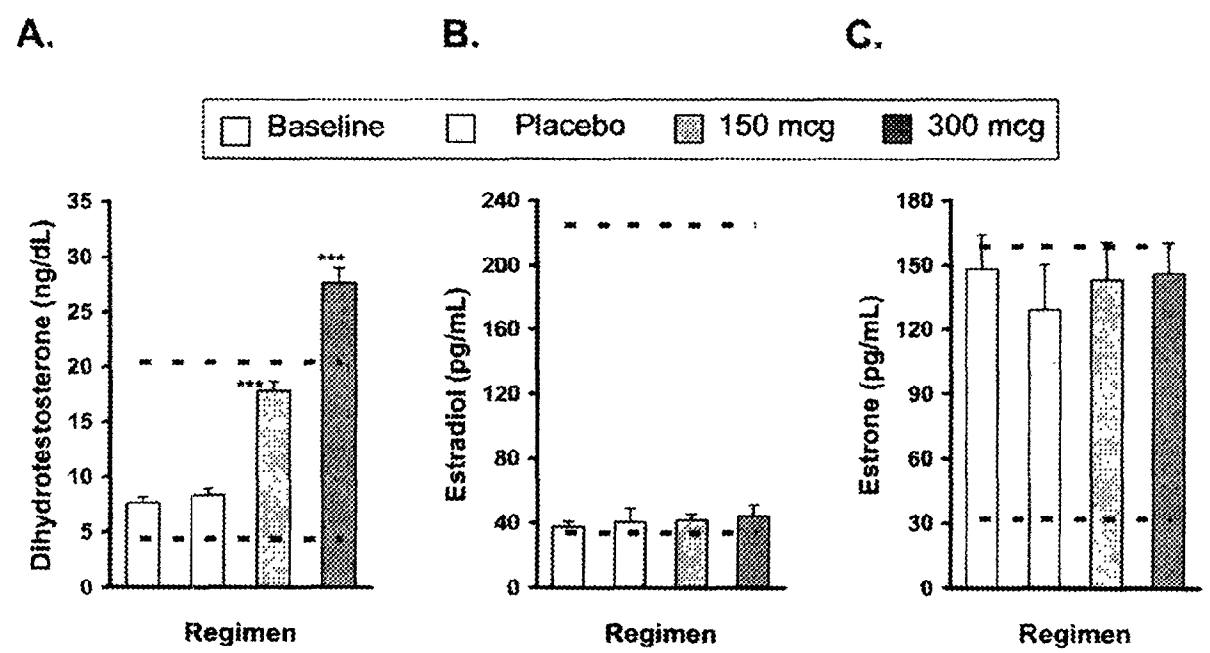

Figure 7. Mean (± SEM) hormone levels measured at baseline and during treatment with experimental transdermal testosterone patches at doses of 0 (placebo), 150 and 300 μg/day from study of 75 oophorectomized with impaired sexual function. (A) Dihydrotestosterone levels. (B) Estradiol levels.(C) Estrone levels. All patients received oral CEEs at doses of 0.625 mg/day or greater. Dashed lines represent normal reference ranges in healthy premenopausal women. ***, $P < 0.001$ vs. placebo. Data from Shifren et al (13). (C) Reprinted with permission from Mazer (1).

levels of SHBG from oral ET (Fig. 6C). The concentrations of DHT also increased into the supra-physiological range (Fig. 7A) as a consequence of the elevated SHBG levels. Had they been measured, free DHT concentrations would likely have remained within the normal range. Estradiol and estrone concentrations did not change with testosterone treatment and remained within the normal range (Fig. 7 B and C), which is consistent with the minimal degree of aromatization from testosterone.

Sexual function was assessed with the Brief Index of Sexual Functioning in Women (BISF-W), a 22-item questionnaire that provides scores pertaining to various aspects of female sexuality as well as an overall composite score (38). The mean BISF-W composite score, expressed as a percentage of the mean values for normal women (38), increased from 52% at baseline to 72% during placebo treatment, 74% during 150 μg/day treatment, and 81% during 300 μg/day treatment (P = .05 for comparison to placebo) (Fig. 8). Likewise, the scores for frequency of sexual activity and pleasure/orgasm increased in a dose-dependent manner and were significantly greater than placebo at the 300 μg/day dose (P = .03). At the higher dose, the percentages of women who had sexual fantasies, masturbated, or engaged in sexual intercourse at least once a week increased 2-3 times from baseline. Scores on the Psychological General Well-being Index also increased significantly at the higher dose.

Safety assessments showed that the experimental testosterone matrix patches were well tolerated on the skin and were not associated with clinically important changes in acne or hirsutism. Lipids, hepatic enzymes and carbohydrate parameters were unaffected by transdermal testosterone, consistent with the absence of a hepatic first-pass effect.

Treatment of AIDS Wasting Syndrome

Miller et al.(12) conducted a 12-week phase II, placebo-controlled, dose-ranging pilot study evaluating the experimental testosterone matrix patch in HIV-infected women with the AIDS wasting

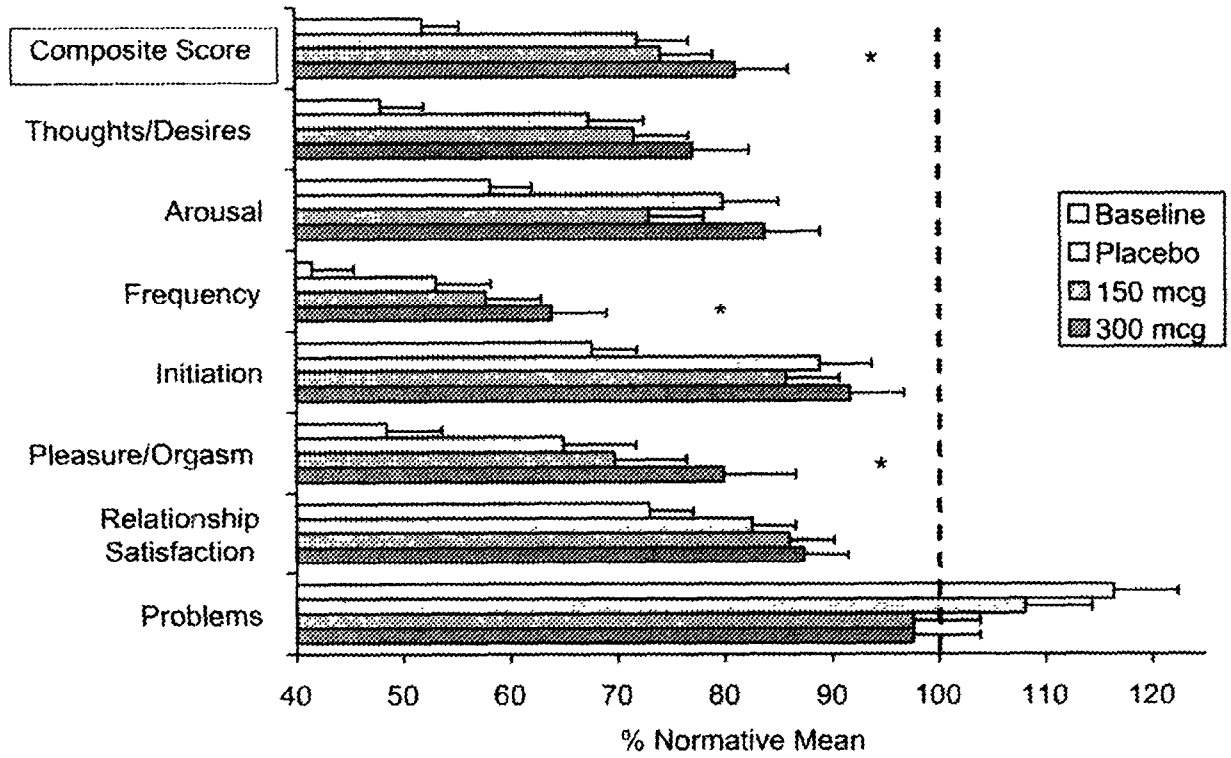

Figure 8. Mean (± SEM) composite and dimension scores on the Brief Index of Sexual Functioning for Women measured at baseline and during treatment with experimental transdermal testosterone patches at doses of 0 (placebo), 150 and 300 μg/day from study of 75 oophorectomized with impaired sexual function. All patients received oral CEEs at doses of 0.625 mg/day or greater. Data are expressed as percentages of the mean values of normal women. *, P < 0.05 vs. placebo. Reprinted with permission from Mazer (1).

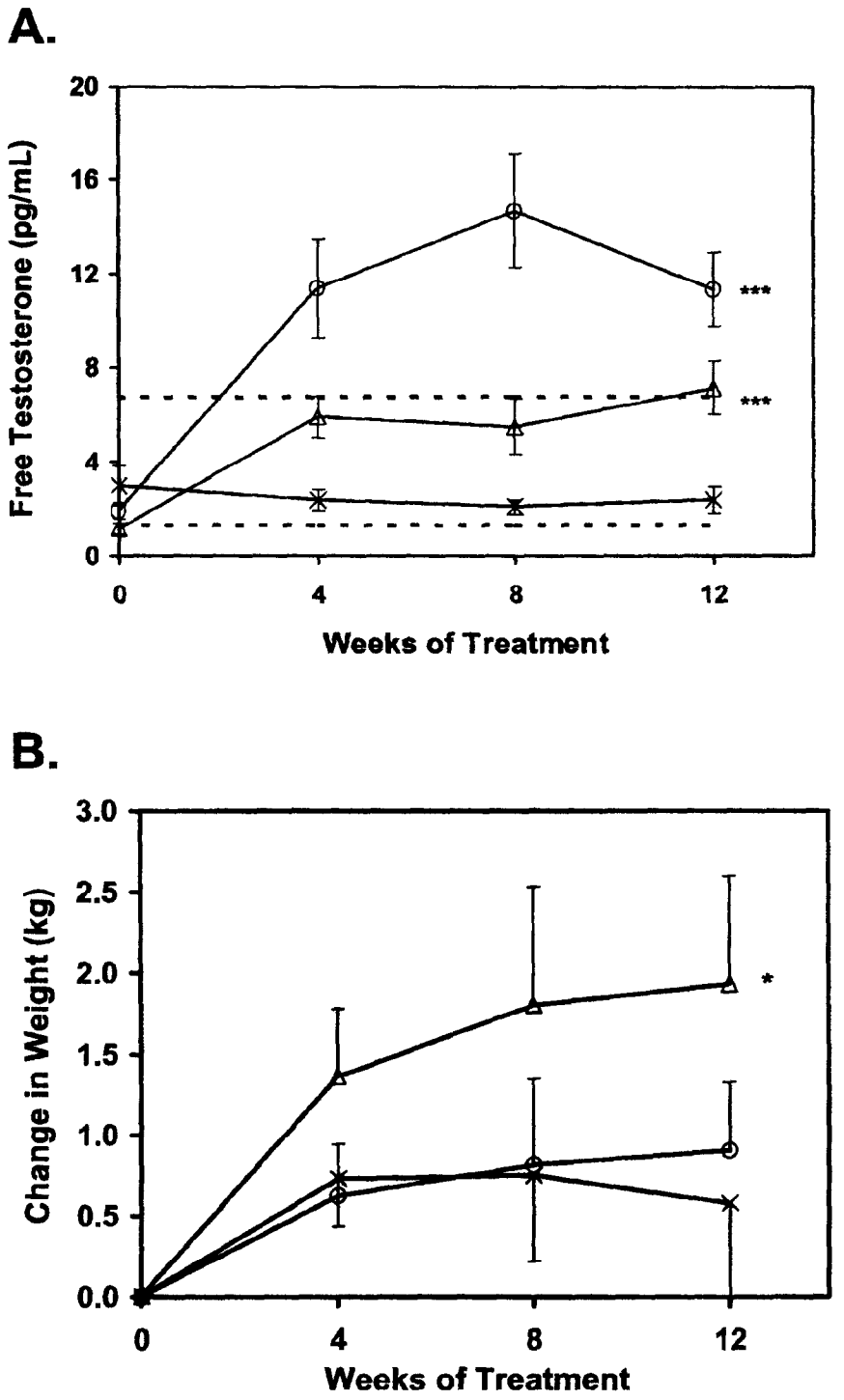

Figure 9. Free testosterone concentrations (A) and change in weight (B) during 12 weeks of experimental testosterone matrix patch treatment in HIV-infected women, by treatment group. X, placebo regimen; Δ, 150 μg/day active regimen; O, 300 μg/day active treatment regimen. *, $P < 0.05$, *** $P < 0.001$ vs. placebo. Adapted with permission from K. Miller, C. Corcoran, C. Armstrong, et al, Transdermal testosterone administration in women with acquired immunodeficiency syndrome wasting: A pilot study, J Clin Endocrinol Metab 1998:83(8);2717-2725. ©The Endocrine Society (12).

syndrome and low testosterone levels. Three parallel groups of patients applied patches twice weekly on the abdomen delivering either 0 (placebo), 150 or 300 μg/day testosterone, respectively. Free testosterone concentrations in the placebo group remained constant at approximately 2-3 pg/ml, whereas levels in the 150 and 300 μg/day groups increased in a dose-proportional manner to average values of 5.9 and 12.4 pg/ml, respectively, exceeding the upper limit of the normal reference range for the 300 μg/day group (Fig. 9A). The increases in free testosterone observed in the 150 and 300 μg/day groups were approximately two-fold greater than projected from the pharmacokinetic study in oophorectomized women (Fig. 2B) and may have resulted from increased absorption, altered metabolism or decreased testosterone clearance in the HIV-infected women. The effect of the testosterone patch treatment on body weight is shown in Fig. 9B. For the lower dose group, an average increase of 1.9 kg was observed after 12 weeks, whereas for the placebo and higher dose groups the increases were 0.6 and 0.9 kg, respectively ($P < .05$ for 150 μg/day vs. placebo). Quality of life indices (RAND 36 Health Survey) also improved to the greatest degree in the 150 μg/day group. The patches were well-tolerated locally and systemically, with no adverse effects on hepatic or lipid parameters in these HIV-infected patients. The finding that the lower dose group achieved better clinical responses than the higher dose group may have been related to the more physiological values of free testosterone achieved with that dosing regimen.

CONCLUSIONS

As in men, transdermal testosterone delivery has a compelling biopharmaceutical rationale for the treatment of women requiring androgen therapy. Moreover, the much lower dosing requirement in women can be achieved with a multi-day matrix patch that is lighter, thinner and better tolerated than the 24-hour testosterone reservoir patches used by men. At present, the testosterone matrix patch is furthest advanced in clinical development and appears to provide more physiological concentrations and profiles of testosterone and its DHT metabolite than currently studied compounded topical gels containing micronized testosterone. In contrast to transdermal testosterone delivery in men, which substantially raises the concentration of circulating estradiol (39, 40), a negligible effect on circulating estradiol concentrations is observed during transdermal testosterone delivery in women. On the other hand, by markedly raising SHBG levels, the concomitant use of oral estrogen therapy increases the total and decreases the free concentrations of testosterone achieved with the testosterone matrix patch. Transdermal estrogen therapy does not alter SHBG concentrations and does not seem to influence testosterone levels in this way.

Phase II placebo-controlled studies reported with the experimental testosterone matrix patch indicate beneficial effects on sexual function and mood in oophorectomized women, and improvements in body weight and quality of life in HIV-infected women with low androgen levels. Although these studies have been relatively short term (3-9 months), the safety data reported to date have shown excellent local tolerability of the patches, no virilizing effects, and no adverse biochemical effects on lipids, hepatic enzymes and carbohydrate parameters.

Although larger phase III trials are needed to confirm the efficacy and safety findings reported thus far, transdermal testosterone therapy seems to be a promising new approach for providing physiologically based treatment of women requiring androgen therapy.

Acknowledgments—This research was supported in part by a grant from Procter & Gamble Pharmaceuticals.

REFERENCES

1. Mazer NA. Testosterone deficiency in women: Etiologies, diagnosis, and emerging treatments. Int J Fertil Women's Med 2002;47(2):77-86.
2. Bachmann G, Bancroft J, Braunstein G, et al. Female androgen insufficiency: The Princeton consensus statement on definition, classification, and assessment. Fertil Steril 2002;77(4): 660-665.
3. Davis SR. When to suspect androgen deficiency other than at menopause. Fertil Steril 2002;77(Suppl 4):68-71.
4. Shifren JL. Androgen deficiency in the oophorectomized woman. Fertil Steril 2002;77(Suppl 4):60-62.
5. Atkinson LE, Chang Y-L and Snyder PJ. Long-term experience with testosterone replacement through scrotal skin. In: Testosterone: Action, Deficiency, Substitution, 2nd Edition. Nieschlag E, Behre HM (eds). Berlin: Springer-Verlag, 1998; pp 365-388.
6. Meikle AW. A permeation-enhanced non-scrotal testosterone transdermal system for the treatment of male hypogonadism. In: Testosterone: Action, Deficiency, Substitution, 2nd Edition. Nieschlag E, Behre HM (eds). Berlin: Springer-Verlag, 1998; pp 389-422.
7. Swerdloff RS, Wang C, Cunningham G, et al. Long-term pharmacokinetics of transdermal testosterone gel in hypogonadal men. J Clin Endocrinol Metab 2000;85(12):4500-4510.
8. Rovati LC, Schmid K, Giacovelli G, et al. Efficacy on climac-

teric symptoms of a new estradiol transdermal patch with active matrix in comparison with a reference reservoir patch. Two long-term randomized multicenter parallel-group studies. Arzneimittelforschung 1999;49(11):933-943.
9. Stevenson JC. Optimising delivery systems for HRT. Maturitas 1999;33(Suppl 1):S31–S38.
10. Bowen AJ, Caramelli KE, Mazer NA. Physiological testosterone replacement for women: pharmacokinetics of experimental transdermal matrix patches in surgically menopausal subjects. In: 80th Annual Meeting of the Endocrine Society; June 24-27, 1998; New Orleans, LA. Abstract P3–P59.
11. Javanbakht M, Singh AB, Mazer NA, et al. Pharmacokinetics of a novel testosterone matrix transdermal system in healthy, premenopausal women and women infected with the human immunodeficiency virus. J Clin Endocrinol Metab 2000;85(7): 2395-2401.
12. Miller K, Corcoran C, Armstrong C, et al. Transdermal testosterone administration in women with acquired immunodeficiency syndrome wasting: A pilot study. J Clin Endocrinol Metab 1998;83(8):2717-2725.
13. Shifren JL, Braunstein GD, Simon JA, et al. Transdermal testosterone treatment in women with impaired sexual function after oophorectomy. N Engl J Med 2000;343(10):682-688.
14. Baylor College of Medicine, Department of Obstetrics and Gynecology, Division of Reproductive Endocrinology and Infertility, Clinical Trials (website), Testosterone Patch Study for Female Sexual Dysfunction. http: //www.baylorart.com/Research/ClinicalTrial.cfm.
15. Grinspoon SK. AIDS Wasting in Women. Anabolic Effects of Testosterone, Massachusetts General Hospital, Boston, MA. http://clinicaltrials.gov/show/NCT00006158.
16. Bhasin S. Phase II randomized study of physiologic testosterone replacement in premenopausal, HIV-positive women. Charles R. Drew University of Medicine and Science, Los Angeles, CA. http://clinicaltrials.gov/show/NCT00004400.
17. Slater CC, Souter I, Zhang C, et al. Pharmacokinetics of testosterone after percutaneous gel or buccal administration. Fertil Steril 2001;76(1):32-37.
18. Mazer NA. Enhanced transdermal delivery of testosterone using the TheraDerm TDS. Topics in Pharmaceutical Sciences 1991: Proceedings of the 51st International Congress of Pharmaceutical Sciences of F.I.P., Washington, D.C., September 1-6, 1991. Crommelin DJA and Midha KK, eds. Stuttgart: Medpharm Scientific Publishers. 1992; pp 149-170.
19. Griffin JE, Allman DR, Durrant JL, et al. Variation in steroid 5 alpha-reductase activity in cloned human skin fibroblasts. Shift in phenotypic expression from high to low activity upon subcloning. J Biol Chem 1981;256(8):3662-3666.
20. Svenstrup B, Brunner N, Dombernowsky P, et al. Comparison of the effect of cortisol on aromatase activity and androgen metabolism in two human fibroblast cell lines derived from the same individual. J Steroid Biochem 1990;35(6):679-687.
21. Longcope C. Androgen and estrogen conversion ratios in aging women. Maturitas 1980;2(1):13-17.
22. Longcope C, Kato T, Horton R. Conversion of blood androgens to estrogens in normal adult men and women. J Clin Invest 1969;48(12):2191-2201.
23. Ahmed SR, Boucher AE, Manni A, et al. Transdermal testosterone therapy in the treatment of male hypogonadism. J Clin Endocrinol Metab 1988;66(3):546-551.
24. Mazer NA, Heiber WE, Moellmer JF, et al. Enhanced transdermal delivery of testosterone: a new physiological approach for androgen replacement in hypogonadal men. J Contr Rel 1992;19:347-362.
25. Bowen AJ, John VA, Ramirez ME, et al. Bioavailability of oestradiol from the Alora (0.1 mg/day) oestradiol matrix transdermal delivery system compared with Estraderm (0.1 mg/day). J Obstet Gynaecol 1998;18(6):575-580.
26. Simon JA. Safety of estrogen/androgen regimens. J Reprod Med 2001;46(3 Suppl):281-290.
27. Arafah BM. Decreased levothyroxine requirement in women with hypothyroidism during androgen therapy for breast cancer. Ann Intern Med 1994;121(4):247-251.
28. Casson PR, Carson SA, Buster JE. Testosterone delivery systems for women: Present status and future promise. Semin Reprod Endocrinol 1998;16(2):153-159.
29. Mazer NA. New clinical applications of transdermal testosterone delivery in men and women. J Control Release 2000;65(1-2):303-315.
30. Southren AL, Gordon GG, Tochimoto S. Further study of factors affecting the metabolic clearance rate of testosterone in man. J Clin Endocrinol Metab 1968;28(8):1105-1112.
31. Longcope C. Androgen metabolism and the menopause. Semin Reprod Endocrinol 1998;16(2):111-115.
32. Vermeulen A, Ando S. Metabolic clearance rate and interconversion of androgens and the influence of the free androgen fraction. J Clin Endocrinol Metab 1979;48(2):320-326.
33. Abraham GE, Lobotsky J, Lloyd CW. Metabolism of testosterone and androstenedione in normal and ovariectomized women. J Clin Invest 1969;48(4):696-703.
34. Chetkowski RJ, Meldrum DR, Steingold KA, et al. Biologic effects of transdermal estradiol. N Engl J Med 1986;314(25): 1615-1620.
35. Longcope C, Hui SL, Johnston CC, Jr. Free estradiol, free testosterone, and sex hormone-binding globulin in perimenopausal women. J Clin Endocrinol Metab 1987;64(3):513-518.
36. Mazer NA. Effect of ERT on testosterone levels in women: Differences between oral and transdermal routes of administration. OBG Management 2001;Sept(Suppl):10-13.
37. Rosario-Jansen T, Mazer NA. Administration of non-oral androgenic steroids to women. International Application Published Under the Patent Cooperation Treaty (PCT), WO 00/76522 A1, International Publication Date 21.12.2000.
38. Mazer NA, Leiblum SR, Rosen RC. The brief index of sexual functioning for women (BISF-W): A new scoring algorithm and comparison of normative and surgically menopausal populations. Menopause 2000;7(5):350-363.
39. Meikle AW, Arver S, Dobs AS, et al. Pharmacokinetics and metabolism of a permeation-enhanced testosterone transdermal system in hypogonadal men: Influence of application site—A clinical research center study. J Clin Endocrinol Metab 1996;81(5):1832-1840.
40. Dobs AS, Meikle AW, Arver S, et al. Pharmacokinetics, efficacy, and safety of a permeation-enhanced testosterone transdermal system in comparison with bi-weekly injections of testosterone enanthate for the treatment of hypogonadal men. J Clin Endocrinol Metab 1999;84(10):3469-3478.

ELSEVIER

Maturitas 34 (2000) S25–S33

MATURITAS
THE EUROPEAN MENOPAUSE JOURNAL

www.elsevier.com/locate/maturitas

Recent insights into the varying activity of estrogens

Michael Dey [a,*], C. Richard Lyttle [b], James H. Pickar [c]

[a] *Wyeth-Ayerst Womens Health Research, 145 King of Prussia Road, Radnor PA 19087, USA*
[b] *Department of Pharmacology, Duke University Medical Center, Durham, NC, USA*
[c] *Wyeth-Ayerst Research, 145 king of Prussia Road, Radnor, PA 19087, USA*

Abstract

Recent advances have added substantially to our understanding of the biology of estrogens. Estrogens are no longer considered to differ only in potency. Two estrogens can have similar effects in one tissue and very different effects in another. Additionally, an estrogen can have different effects in different tissues. It is now recognized that there are at least two estrogen receptors, ER-α and ER-β, and that it is quite likely that estrogens also work through non-genomic mechanisms. The development of new methods of chromatographic separation has aided substantially in our ability to characterize the composition of Premarin, including the identification of estradiene, the fourth-most abundant estrogen in Premarin. Recent studies have contributed to our understanding of the unique profile of $\Delta^{8,9}$ dehydroestrone sulfate, another of the Premarin estrogens. It was found that $\Delta^{8,9}$ dehydroestrone sulfate is an active estrogen with a distinct pharmacological profile that results in significant clinical activity in vasomotor, neuroendocrine and bone preservation parameters. However, it displayed little or no efficacy, at the dose studied, on other peripheral parameters normally affected by classical estrogens. Increasing knowledge of the unique profiles of the Premarin components, as well as their complex interaction, will help to increase our understanding of the clinical profile of Premarin.

Keywords: Estrogen replacement thearpy; Reproductive hormones; Dementia

1. Introduction

Technological advances have dramatically increased our knowledge of estrogen replacement therapy. Although estrogens have been on the market in the United States for over 58 years, researchers continue to discover more and more about their effects on various tissues. Unlike most areas of pharmaceutical research, in which new discoveries come out of the laboratory, most of the discoveries involving estrogens have been gained from clinical practice, sending pharmaceutical companies back to the laboratory in an effort to understand the mechanisms by which these estrogens produce their effects. In the past, estrogens were considered strictly reproductive hormones. It is now apparent that estrogens not only affect the reproductive tissues but also affect the bone [1], the vascular system [2], and the brain, both in relation to vasomotor symptoms [3]

* Corresponding author. Tel.: +1-610-9024050; fax: +1-610-9645683.
E-mail address: deym@labs.wyeth.com (M. Dey)

0378-5122/00/$ - see front matter
PII: S0378-5122(00)00110-9

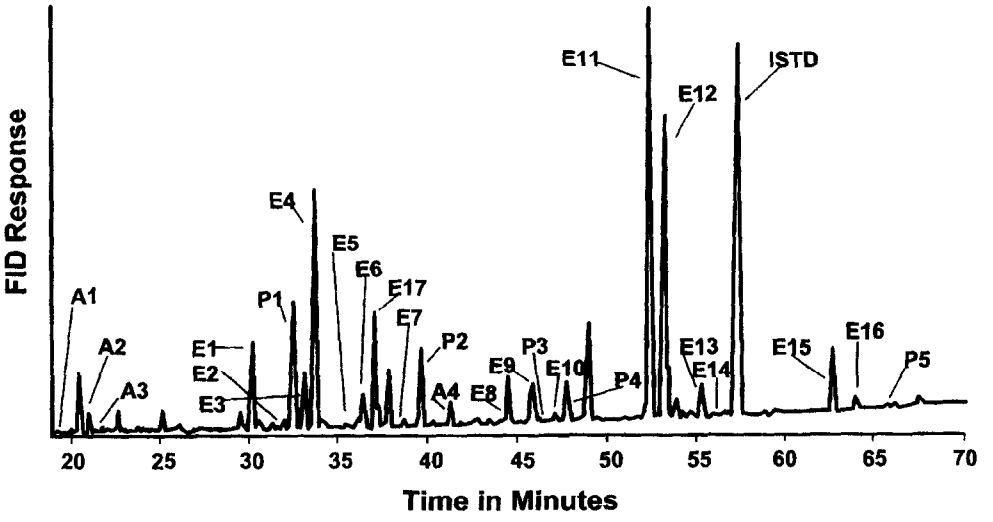

Fig. 1. Chromatogram that identifies the complex mixture of estrogenic (E), progestogenic (P), and androgenic (A) components of Premarin.

and, reportedly, to the prevention of dementia [4,5]. Estrogens have also been reported to reduce the risk of macular degeneration [6], and an American Cancer Society study indicated that estrogens were important in the prevention of colon cancer [7]. As the concept of estrogens has changed, one of the fundamental questions that has arisen is how an estrogen working through a single estrogen receptor can have such a wide variety of different effects. This article explains how advances in analytical technology and molecular endocrinology have widened our understanding of the mechanisms of estrogen and, as a result, of estrogen replacement therapy.

2. Components in conjugated estrogens (Premarin)

Premarin®[1] (conjugated estrogens tablets, USP) is a natural product derived exclusively from the urine of pregnant mares. When Premarin was first approved in the United States in 1942, it was believed to contain principally two estrogens, estrone and equilin, along with others not identified at that time [8]. The reason for this was that the analytical method used, the Marrian–Kober reaction [9], involved a colorimetric procedure that gave investigators two sets of peaks, one corresponding to estrone, one to equilin. Unbeknownst to investigators, all of the other components in Premarin were buried under these two peaks. It was not until the development of the packed column gas chromatographic method in 1972 that Premarin was shown to be made up of a number of other components [10]. Since then, Premarin has been thought to be comprised of at least 10 estrogens, estrone comprising about 50%, equilin comprising about 22%, and the others — eg delta^{8,9}-dehydroestrone, a newly researched estrogen which is discussed in more detail below — comprising the remaining 27% [11]. Thus, thanks to further advances in technology, we now know that Premarin is far more complex than originally believed. High-pressure liquid chromatography/mass spectrometry has revealed not only estrogens but also androgens and progestins in Premarin [12] (Fig. 1).

Determining the composition of Premarin is important because all estrogens are not the same: an estrogen can have different effects in different tissues, and two estrogens can have similar effects in one tissue but very different effects in another [13]. These data are now well established. In 1975, Beck and Friedrich compared equilin to Premarin [14]. Using relief of vasomotor symptoms and changes in the vaginal epithelium as clinical end

[1] Wyeth-Ayerst Laboratories, St Davids, PA.

points, they concluded that equilin was two and a half times more potent than Premarin. In 1988, however, Lobo et al. concluded that equilin was only half as potent as Premarin when urinary calcium excretion was used as the clinical end point [15]. Thus, dramatically different conclusions about the relative activity and relative potency of various estrogens are possible depending upon the end point or the tissue being considered.

3. Action of estrogens

Today, the fact that estrogens can have a wide range of effects is commonly known. An estrogen can be a full agonist such as 17 β-estradiol, or they can be a full antagonist such as the ICI compounds [13]; in between are the mixed function compounds, such as tamoxifen and raloxifene, which are partial agonists, partial antagonists, depending upon the tissue being analyzed (Fig. 2) [16]. 17 β-estradiol, for example, stimulates in each of three tissues, the uterus, the bone, and the breast, whereas the ICI compound has no effect in any of the tissues [11,13]. In the case of tamoxifen and raloxifene, both stimulate in the uterus (although the potency changes such that tamoxifen stimulates the uterus more than raloxifene); both stimulate in bone (although while in the bone, raloxifene is a better stimulator or protector of bone loss than tamoxifen); and both tamoxifen and raloxifene appear to be antagonists or growth inhibitors in the breast [13]. It is likely that many of the Premarin components fall into this mixed function group [17].

With these advances in our knowledge, three central tenets of estrogen action have become outdated: first, that the biological activity of an estrogen receptor ligand is directly proportional to its binding affinity to the receptor; second, that all estrogens are functionally the same and when corrected for this affinity are indistinguishable; and third, that the estrogen receptor works in a vacuum. This article will discuss these in more detail.

It is no longer accepted that an estrogen's receptor-binding activity predicts its biological activity. When the 10 estrogens originally shown in Premarin were ranked according to their binding affinity to the human estrogen receptor, the estrogen with the highest binding affinity was 17 β-estradiol, followed by 17 β-dihydroequilin, and so on (Fig. 3) [18]. Of interest in this case is the fact that delta^{8,9}-dehydroestrone, which ranked ninth

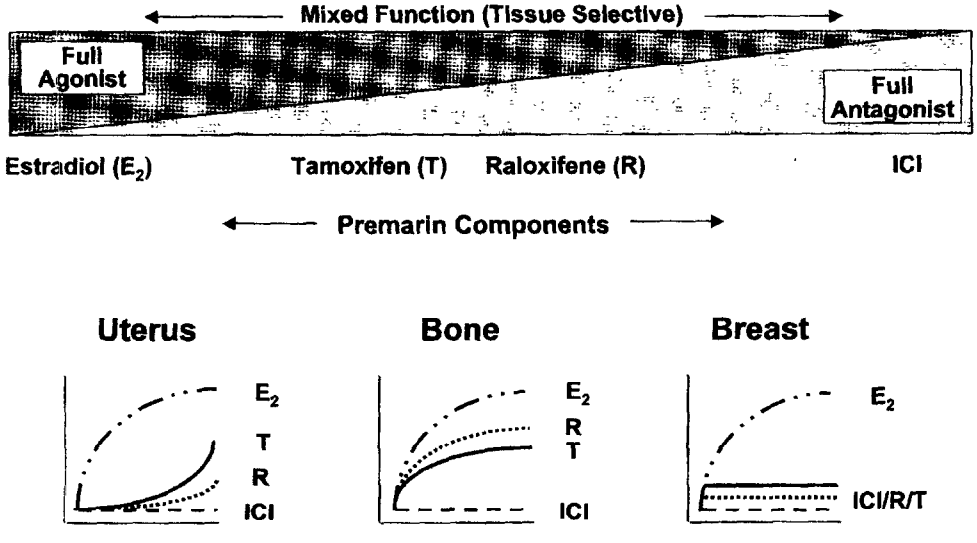

Fig. 2. The status of different setrogenic compounds as agonists or antagonists. Estrogens can be full agonists (estradiol), partial agonist/antagonist (tamoxifen, raloxifene), or full antagonists (ICI). The estrogenic effects of these compounds on different tissues are illustrated in the three graphs.

Rank	Human ER Binding	Gene Activation C-3 Promoter	Gene Activation Consensus ERE
1.	17β-Estradiol	17β-Estradiol	17β-Estradiol
2.	17β-Dihydroequilin	$\Delta^{8,9}$-Dehydroestrone	Estrone
3.	17β-Dihydroequilenin	Estrone	$\Delta^{8,9}$-Dehydroestrone
4.	17α-Dihydroequilin	17β-Dihydroequilenin	Equilin
5.	17α-Estradiol	Equilenin	17β-Dihydroequilin
6.	Estrone	17β-Dihydroequilin	17β-Dihydroequilenin
7.	Equilin	Equilin	17α-Dihydroequilin
8.	17α-Dihydroequilenin	17α-Dihydroequilin	17α-Estradiol
9.	$\Delta^{8,9}$-Dehydroestrone	17α-Dihydroequilenin	Equilenin
10.	Equilenin	17α-Estradiol	17α-Dihydroequilenin

Fig. 3. Relative binding affinity and potency of receptor activation of different Premarin components.

out of 10 in terms of binding affinity, ranked second only — after 17 β-estradiol — in its ability to produce a biological effect, i.e. to activate a gene (in this case the C3 promoter). Clearly, its binding affinity did not reflect its ability to turn on the C3 promoter. In the case of the consensus estrogen response element (ERE), delta^{8,9}-dehydroestrone's activity ranked third, behind 17 β-estradiol and estrone [18]. Again this was not reflected by its binding affinity; nor, in fact, was that of many other estrogens. Thus, depending upon the gene, we see in fact the binding affinity does not necessarily predict biological activity.

4. Clinical trials

It is one thing to look at receptor-binding activity and compare an estrogen's ability to activate genes, another to ask the fundamental question of whether this model is predictive of what happens clinically. Two similar trials examined the clinical effects of delta^{8,9}-dehydroestrone [19,20]. For the sake of space, only one trial's results will be discussed here [19]. In that trial, 30 postmenopausal women were divided randomly into three treatment groups, 10 women per group. One group received estrone sulfate at 1.25 mg/d, the second group received delta^{8,9}-dehydroestrone sulfate at 0.125 mg/d (one tenth the dose of estrone sulfate), and the third group received the two drugs combined at the same respective doses. Estrone was put into the study as a positive control. Estrone sulfate is a component of Premarin and a dose of 1.25 mg/d is known to be effective in preventing bone loss. The dose of estrone sulfate was used to set the dose for delta^{8,9}-dehydroestrone sulfate to keep both drugs in the same relative proportion that they are present in a Premarin tablet.

A number of different clinical end points were examined. The first end point was urinary excretion of *N*-telopeptide, an important marker for bone resorption. Levels of urinary *N*-telopeptide increase in women after menopause, reflecting an increase in bone resorption. Replacing a woman's estrogens leads to a decrease in the excretion of *N*-telopeptide, an effect seen in this trial, in which there was a significant, 40% reduction in urinary *N*-telopeptide with 1.25 mg of estrone [19]. This is consistent with estrone sulfate's effectiveness in the prevention of bone loss at a dose of 1.25 mg. Delta^{8,9}-dehydroestrone sulfate at one tenth that dose (0.125 mg/d) produced an identical and significant 40% reduction in urinary *N*-telopeptide, and delta^{8,9}-dehydroestrone sulfate and estrone sulfate combined also produced a 40% reduction. Thus there was no additive effect of the two together. Clearly, in terms of its ability to prevent bone resorption, delta^{8,9}-dehydroestrone sulfate appears to be a very potent estrogen.

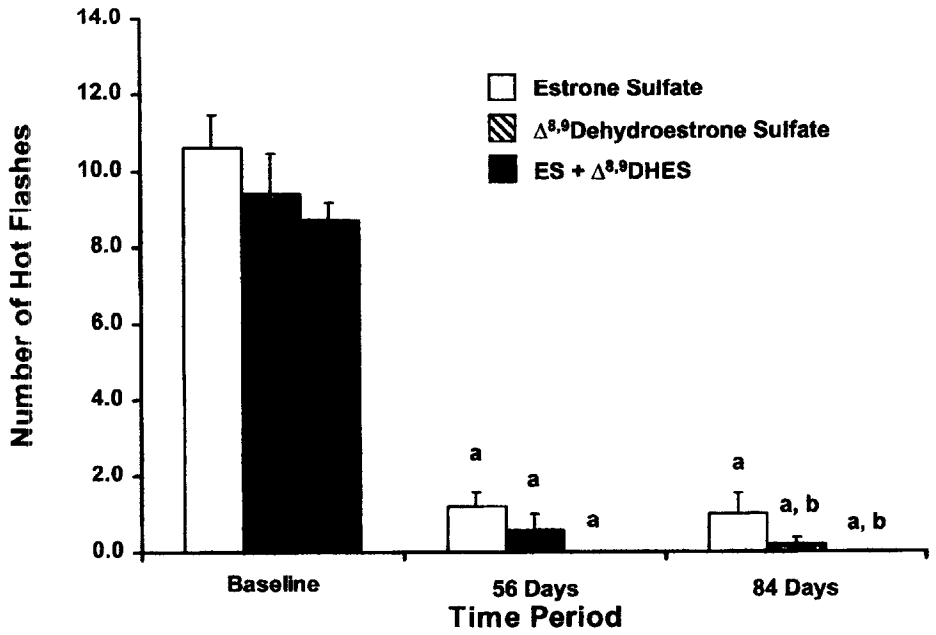

Fig. 4. Effect of estrone sulfate and delta^{8,9}-dehydroestrone sulfate on the number of hot flushes per day in postmenopausal women. Data represent the mean ± SEM of five patients per group. $^{a}P < 0.001$ versus respective baseline value; $^{b}P < 0.005$ versus the estrone sulfate-treated group. DHES, dehydroestrone sulfate.

The same trial evaluated the effects of delta^{8,9}-dehydroestrone on symptoms, the second end point, specifically the number and severity of hot flushes. In those women receiving 1.25 mg of estrone, a significant reduction occurred in the number of hot flushes per day (Fig. 4) [19]. A similar, significant reduction in the number of hot flushes occurred with 0.125 mg of delta^{8,9}-dehydroestrone rate when the two estrogens were combined, there was an almost complete resolution of the hot flushes. Similar results were found for the severity of symptoms.

The third end point was biochemical markers of estrogen action. Serum levels of follicle stimulating hormone (FSH) declined 40–50% with 1.25 mg of estrone sulfate (Fig. 5) [19]. A similar effect of 0.125 mg of delta^{8,9}-dehydroestrone was found, although it appears to have been slower. When both estrone and delta^{8,9}-dehydroestrone were combined, there was a slight increase in suppression of FSH. Again, delta^{8,9}-dehydroestrone here was present at one tenth the dose of estrone. Investigators found similar results for luteinizing hormone (LH): estrone produced approximately a 20% reduction in circulating levels of LH as did

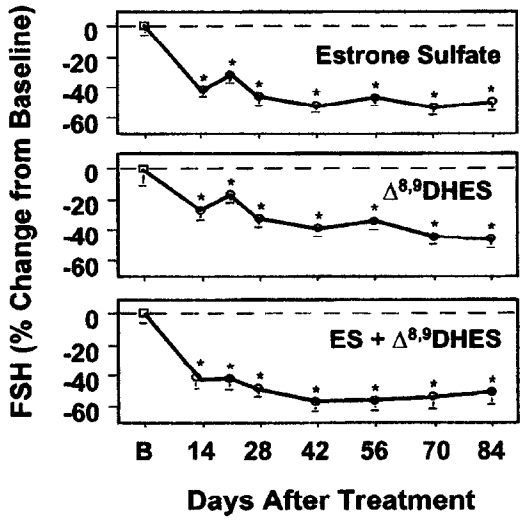

Fig. 5. Effect of esterone sulfate and delta^{8,9}-dehydroestrone sulfate on serum follicle-stimulating hormone levels in postmenopausal women. Data represent the mean + SEM of ten patients per group. Open squares indicate FSH levels before treatment initation. Solid symbols indicate FSH levels (percent change from baseline) at different times during therapy. Astericks denote statistically significant differences compared to baseline ($P < 0.05$). B, baseline; ES, estrone sulfate; DHES, dehydroestrone sulfate.

studied endothelial cells grown in culture and exposed to various conditions. When endothelial cells were then exposed to oxidized LDL alone, 80% died, or, in other words, 20% survived [24]. With the addition of the estrogens (at the same concentration) to cells exposed to oxidized LDL, each estrogen enhanced cell survival beyond that of those exposed to oxidized LDL alone. Even though 17 β-estradiol doubled the survival over the cells exposed only to oxidized LDL, so that the survival went from 20 to 40%, other estrogens were significantly more effective; several of them enhanced survival beyond the control, resulting in better than 100% survival. It is important to note that, although the estrogens were able to enhance survival of the endothelial cells directly exposed to oxidized LDL, there was nevertheless a wide range in their abilities to do this. Delta^{8,9}-dehydroestrone was the most effective, several times more effective than 17 β-estradiol or estrone.

6. Recent components identified in Premarin

One of the newest estrogens in Premarin being characterized by investigators is estradiene, the fourth most abundant estrogen in Premarin. It is present in amounts about three times greater than that of delta^{8,9}-dehydroestrone and has activity similar to delta^{8,9}-dehydroestrone. In pre-clinical study models that studied its effect on total cholesterol, an increase in the dose was associated with a corresponding decrease in total plasma cholesterol [24]. In the case of the uterus, an increase in dose was associated with an increase in uterine weight in animals [24]. Thus estradiene's activity in these two tissues is very similar to that of delta^{8,9}-dehydroestrone.

7. Tissue specificity of estrogens

The issue that arises from these recent data is how these estrogens can differ from one another with regard to the varying activity from tissue to tissue. The answer to this question may lie in our knowledge of the estrogen receptor and what we understand to be the mechanism by which the estrogens activate the genes. In the early 1970s, a common concept to explain this mechanism was the lock and key hypothesis, which in large part was developed using estrogens. It was thought that the estrogens' principal role was to bind to the ligand binding domain of the estrogen receptor and convert that inactive receptor through a conformational change to an active dimer of the receptor. This then enabled that active receptor with its estrogen to bind with the target region of the gene and alter the profile of the cell. An estrogen agoinst was thought to change the shape of the receptor while an antiestrogen was believed to bind to that same site of the receptor and block the estrogen from binding, thus blocking the conformational change of the inactive receptor to its active form.

The problem with this model is that it does not, or cannot, explain the relevant data. One clinical piece that signaled a change in our thinking about this model of how estrogens work was a study conducted by Love et al. [25] published in the New England Journal of Medicine in 1992. Love et al. studied the effect of the antiestrogen, tamoxifen, versus placebo on the loss of spinal bone mineral density in a group of women over 24 months. Tamoxifen is given to women to inhibit the growth of estrogen receptor–dependent breast tumors. Since estrogen was shown to be required for maintenance of bone mineral density, it is likely that Love and colleagues expected that an antiestrogen like tamoxifen would oppose the estrogen's action and that bone quality in those patients would decline. Surprisingly, the resulting data showed that women on tamoxifen, the antiestrogen, not only did not lose bone but actually gained bone. The fact that in this study tamoxifen actually functioned as an estrogen in bone revealed that the simple model of estrogen action cannot be correct. This was a crucial observation. The tamoxifen paradox has taught researchers a number of lessons: that this classical model for estrogen receptor action is incorrect, that the classification of compounds as agonists and antagonists is tissue- and cell-dependent, that the mechanism of action of estrogen action is not the same in all cells, and that it is in fact possible to develop tissue-selective estrogens.

8. Mechanisms of estrogens actions

Three recent discoveries were major breakthroughs in understanding of the mechanism of estrogen action and helped to illuminate further how different estrogens, despite working through the same receptor, can manifest different biologies in different tissues. The first was the discovery by Jan-Åke Gustafsson and his group at the Karolinska Institute in 1995 of a second estrogen receptor, ERβ [26]. The first estrogen receptor, ERα, was identified by Elwood Jensen and Jacn Gorsky in the mid 1960's. ERβ was discovered by Gustafsson, remarkably, in the male rat prostate when he was looking for altered androgen receptors. A number of tissues have now been viewed, revealing that there is a differential localization in many of these tissues for the different estrogen receptors. For example, in the case of the prostate, there is no ERα but there are high levels of ERβ. In contrast, the uterus contains mostly ERα. The ovary contains both ERα and ERβ, although they are located in different cell types in the ovary. In the pituitary, again both ERα and ERβ are expressed; however, ERβ appears to be expressed in different cell types than ERα, which may help explain the difference seen with delta^{8,9}-dehydroestrone in relation to its effects on FSH and LH and its lack of effects on prolactin.

The second major discovery is related to the structural differences in these two receptors. Two different regions are particularly interesting: the DNA binding region and the ligand-binding region. Both estrogen receptors have approximately the same number of amino acids in those two regions [24]. There are 80 and 83 amino acids in the DNA binding domain and 243 and 250 amino acids in the ligand binding domain of ERα and ERβ, respectively. However, a closer look at the sequence of those amino acids reveals that they approach 97% homology in the DNA binding domain, which suggests that these different estrogen receptors bind to the same region of DNA. In the ligand binding domain, however, they are only about 60% homologous, suggesting that they bind different estrogens to different degrees. This has been borne out by estrogen receptor binding data.

A second discovery was made in Donald McDonnells's lab at Duke University by a post-doctoral scientist, David Clemm [27]. As discussed above, the old concept of the estrogen receptor stipulated that the receptor was either in the inactive conformation or the active conformation. Clemm believed that this concept was inaccurate, theorizing that the estrogen receptor could take on a variety of conformations depending on the estrogen it bound. An analogy was the workings of a rheostat on a light switch: instead of either being off or on, it can take on a variety of different levels of lighting in a room or different conformations. Clemm demonstrated that when different estrogens bind to the receptor, they cause the receptor protein to shift into a different conformation. As the different estrogens drive the receptor into different shapes, the different conformations that result may then be the basis for differential levels of activity or tissue selectivity. Furthermore, not all the cells recognize all these shapes identically.

Third and perhaps most important was the discovery, in 1996, of co-activators by Sergio Oñate in Bert O'Malley's lab in Houston [28]. These co-activator or co-repressor proteins are believed to be able to recognize the different shapes of the estrogen receptor complex and facilitate the binding and activation of specific segments of DNA. As a result some cells have co-activators that recognize the shape of the estrogen receptor, other cells have co-activators that recognize any shape of the extrogen receptor complex. In all probability specific co-activator proteins are then expressed in the uterus, the brain, or the bone, tissues that recognize different shape of the estrogen receptor.

9. Summary

According to the model of the estrogen or steroid hormone receptor as we understand it today, an estrogen or ligand migrates through the cell membrane and into the nucleus, where it binds with the receptor, which changes conformation and forms a homo dimer. The resulting ligand-induced conformation then enables

co-activator or co-repessor complex formation which in turn, enables tissue specific gene expression to occur. This model explains how estrogens acting through the same receptor can induce different receptor conformations, resulting in different biologies.

References

[1] Reginster JY, Christiansen C, Dequinze B, et al. Effect of transdermal 17 β-estradiol and oral conjugated equine estrogens on biochemical parameters of bone resorption in natural menopause. Calcif Tissue Int 1993;53:13–6.

[2] Stevenson JC, Crook D, Godsland IF, Collins P, Whitehead MI. Hormone replacement therapy and the cardiovascular system: nonlipid effects. Drugs 1994;47(2):35–41.

[3] Johnson SR. Menopause and hormone replacement therapy. Med Clin North Am 1998;82:297–320.

[4] Kawas C, Resnick S, Morrison A, et al. A prospective study of estrogen replacement therapy and the risk of developing Alzheimer's disease: the Baltimore Longitudinal Study of Aging. Neurology 1997;48:1517–21.

[5] Paganini-Hill A, Henderson VW. Estrogen replacement therapy and risk of Alzheimer disease. Arch Intern Med 1996;156:2213–7.

[6] Eye Disease Case-Control Study Group. Risk factors for neovascular age-related macular degeneration. Arch Ophthalmol 1992;110:1701–8.

[7] Calle EE, Miracle-McMahill HL, Thun MJ, Heath CW. Estrogen replacement therapy and risk of fatal colon cancer in a prospective cohort of postmenopausal women. J Nat Cancer Inst 1995;87:517–23.

[8] Woodcock, J. Memorandum: approvability of a synthetic generic version of Premarin. FDA Center for Drug Evaluation & Research Web site. May 5, 1997. Available at: http://www.fda.gov/cder/news/celetterjw.htm. Accessed December 3, 1997.

[9] Jones HA, Hahnel R. The specificity and mechanism of the Kober reaction. Steroids 1969;13:693–710.

[10] Roman R, Yates CH, Millar JF, Vanden Heuvel WJA. Identification of estrogens isolated from pregnant mares' urine. Can J Pharm Sci 1975;10:8–11.

[11] Dey M. The new science of hormone replacement therapy. J Br Menopause Soc 1999;2:4–5.

[12] Klein R. The composition of Premarin. Int J Fertil 1998;43:223.

[13] Grese TA, Dodge JA. Selective estrogen receptor modulators (SERMs). Curr Pharm Design 1998;4:71–92.

[14] Beck VA, Friedrich F. Equilinsulfat zur substitution beim menopause-syndrom. Wien Klin Wochenschr 1975;87:59–62.

[15] Lobo RA, Nguyen HN, Eggena P, Brenner PF. Biologic effects of equilin sulfate in postmenopausal women. Fertil Steril 1988;49:234–8

[16] Paech K, Webb P, Kuiper GG, et al. Differential ligand activation of estrogen receptors ERα and ERβ at AP1 sites. Science 1997;277:1508–10.

[17] Dodge JA. International Congress of Endocrinology San Francisco 1996;249 (abstract).

[18] Data on file. Wyeth Ayerst Laboratories/Ligand Pharmaceuticals.

[19] Baracat E, Haider M, López FJ, Pickar J, Dey M, Negro-Vilar A. Estrogen activity and novel tissue selectivity of $\Delta^{8,9}$-dehydroestrone sulfate in postmenopausal women. J Clin Endocrinol Metab 1999;84:2020–7.

[20] Bhavnani BR, Cecutti A, Dey MS. Biologic effects of delta-8-estrone sulfate in postmenopausal women. J Soc Gynecol Invest 1998;5:156–60.

[21] Bush TL, Barrett-Connor E, Cowan LD, et al. Cardiovascular mortality and noncontraceptive use of estrogen in women: results from the Lipid Research Clinics Program Follow-up Study. Circulation 1987;75:1102–9.

[22] Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity. New Engl J Med 1989;320:915–24.

[23] Subbiah MTR, Kessel B, Agrawal M, Rajan R, Abplanalp W, Rymaszewski Z. Antioxidant potential of specific estrogens on lipid peroxidation. J Clin Endocrinol Metab 1993;77:1095–7.

[24] Data on file. Wyeth Ayerst Laboratories.

[25] Love RR, Mazess RB, Barden HS, et al. Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. New Engl J Med 1992;326:852–6.

[26] Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson J-Å. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 1996;93:5925–30.

[27] McDonnell DP, Clemm DL, Hermann T, Goldman ME, Pike JW. Analysis of estrogen receptor function in vitro reveals three distinct classes of antiestrogens. Mol Endocrinol 1995;9:659–69.

[28] Oñate SA, Tsai SY, Tsai M-J, O'Malley BW. Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 1995;270:1354–7.

Pergamon

J. Steroid Biochem. Molec. Biol. Vol. 67, No. 2, pp. 119–131, 1998

Printed in Great Britain

PII: S0960-0760(98)00082-X

0960-0760/98 $19.00 + 0.00

Pharmacokinetics and Pharmacodynamics of a Novel Estrogen Δ^8-Estrone in Postmenopausal Women and Men

Bhagu R. Bhavnani*, Anthony Cecutti and Alan Gerulath

Department of Obstetrics and Gynecology, Institute of Medical Sciences, University of Toronto and St. Michael's Hospital, 30 Bond Street, Toronto, Ontario, Canada M5B 1W8

Recently a tenth equine estrogen, identified as the sulfate ester of Δ^8-estrone has been reported to be present in Premarin (a conjugated equine estrogen preparation), and because of its unique ring B unsaturated structure (conjugated double bond in the B ring), we have, in the present study, determined its pharmacokinetics in postmenopausal women and men, its interaction with uterine estrogen receptors and its uterotropic activity. After the administration of [^{14}C]Δ^8-estrone, blood was drawn at various time intervals, and the plasma fractionated into the unconjugated sulfate and glucuronide fractions. The disappearance of radioactivity as Δ^8-estrone from plasma can be described as a function of two exponentials. The half-lives of the first and second components were 5 ± 0.2 and 40.4 min, respectively. The mean metabolic clearance rate calculated (MCR), was 1711 ± 252 l/d m^2. From the unconjugated fraction, Δ^8-17β-estradiol was also isolated and identified. From the sulfate conjugated fraction, Δ^8-estrone sulfate and Δ^8-17β-estradiol sulfate were isolated in almost equal amounts. No other metabolites of Δ^8-estrone was detectable in the plasma. Both Δ^8-estrone and Δ^8-17β-estradiol bind with human endometrial and rat uterine estrogen receptors with high affinity. The binding affinities of Δ^8-17β-estradiol for human endometrial and rat uterine cytoplasmic receptors were 4 and 25 times higher than those of the parent estrogen Δ^8-estrone, respectively. Administration of Δ^8-estrone and Δ^8-17β-estradiol (2 μg/100 g body weight) to immature rats significantly ($P < 0.05$) increased the uterine weight compared to the controls. These data demonstrate that Δ^8-estrone has estrogenic activity, and that it is further metabolized in man to a single more potent estrogen, Δ^8-17β-estradiol. The extent of this activation by 17β-reduction appears to be greater than that observed with other estrogens. Both estrogens circulate as sulfate conjugates and are very slowly eliminated from the circulation. These data further suggest that Δ^8-estrone and its major metabolite Δ^8-17β-estradiol can contribute to the overall *in vivo* biological effects of Premarin.

J. Steroid Biochem. Molec. Biol., Vol. 67, No. 2, pp. 119–131, 1998

INTRODUCTION

Conjugated equine estrogen preparations such as Premarin® (Wyeth–Ayerst, Philadelphia, PA), are widely prescribed for estrogen replacement therapy for prevention of osteoporosis and cardiovascular disease in postmenopausal women. Premarin contains [1] the sulfate esters of the ring B saturated 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), 17β-dihydroequilenin (17β-Eqn; 1,3,5(10)6,8-estra-pentaen-3,17β-diol) and 17α-dihydroequilenin (17α-Eqn; 1,3,5,(10)6,8-estrapentaen-3,17α-diol). Recently, a novel ring B unsaturated estrogen component identified as Δ^8-estrone (Δ^8-E_1, Δ^8-estrone; 3-hydroxy1,3,5(10)8-estratetraen-17-one) has been found in Premarin. The structures of these ten estrogens are shown in Fig. 1. Approx. 3.5% of the total

*Correspondence to B. R. Bhavnani. Tel. 416 8645089; Fax: 416 8645046; e-mail: bhavnani@smh.toronto.on.ca.

Received 16 Feb. 1998; accepted 12 May 1998.

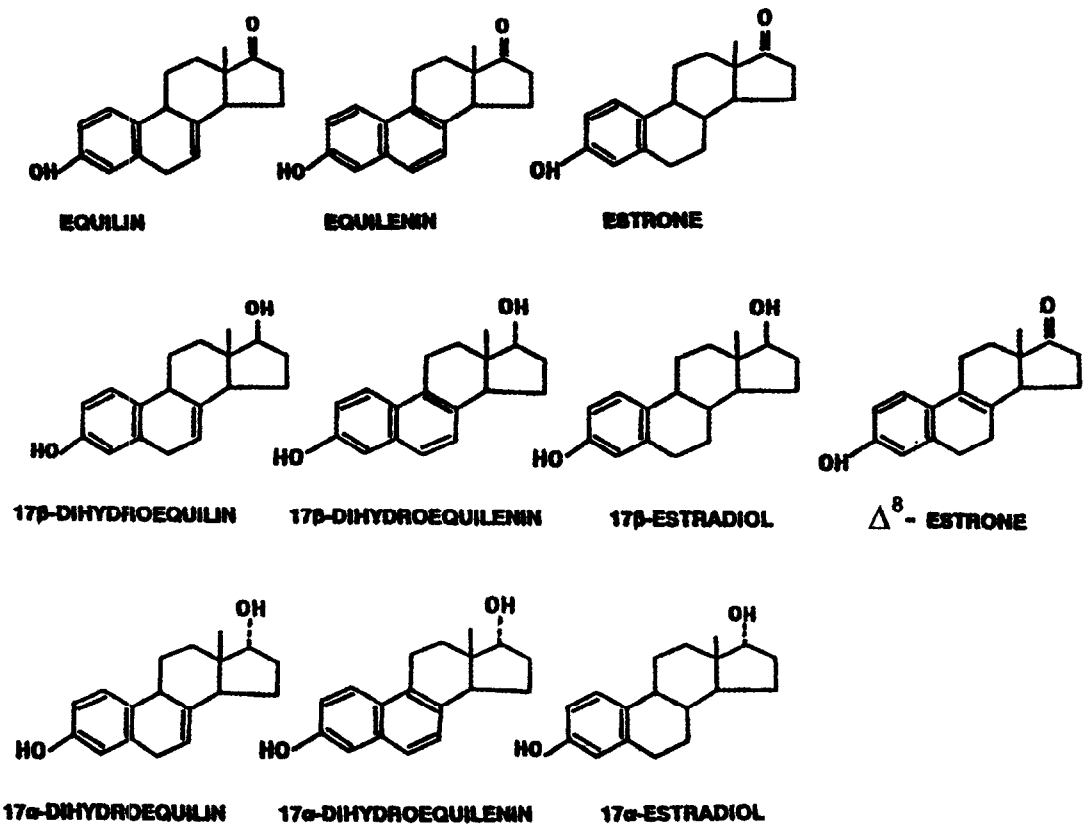

Fig. 1. Structure of estrogens present in pregnant mare's urine. All of these estrogens in their sulfate ester form are present in the drug Premarin.

estrogens present in a Premarin tablet is made up of Δ^8-estrone sulfate (Δ^8-E_1S), and it is the fifth most abundant component. Bhavnani *et al.* [2], reported that small doses (0.125 mg; amount present in one 2.5 mg Premarin tablet) of Δ^8-E_1S, administered to post-menopausal women daily for 8 weeks, decreased the levels of urinary *N*-telopeptides, a marker for bone turnover, and also increased the lag time for the oxidation of low density lipoprotein bound cholesterol (LDL-C). These results suggested that even though Δ^8-E_1S makes up only a small percentage of the total estrogens present in Premarin, it has significant estrogenic activity and it can contribute to the overall beneficial effects on bone and cardiovascular systems ascribed to Premarin.

There is no information available regarding the metabolic fate of Δ^8-E_1 and Δ^8-E_1S in any species, and therefore we decided to investigate the pharmacokinetics of Δ^8-E_1 and Δ^8-E_1S by methods previously described for other ring B unsaturated estrogens [3,4]. To carry out these studies, high specific activity (radioactive) labeled Δ^8-E_1 and Δ^8-E_1S are required. Though we were able to obtain [^{14}C] Δ^8-E_1 and [^{14}C] Δ^8-E_1S via custom synthesis, the high specific activity [^{14}C] Δ^8-E_1S decomposes fairly rapidly and was unsuitable for human experimentation. Similarly, attempts to stabilize the [^{14}C] Δ^8-E_1S by adding large amounts of tris-(hydroxymethyl) aminomethane according to a U.S. patent [5], also resulted in a product that was not suitable for intravenous administration to humans. Therefore, in the present study, the pharmacokinetics of only Δ^8-E_1 were determined by the administration of [^{14}C] Δ^8-E_1 using the single injection technique [3,4,6]. The metabolic clearance rate (MCR) of Δ^8-E_1 was determined and its main metabolites in blood were identified. We have also determined the interaction of Δ^8-E_1 and its main metabolite Δ^8-17β-estradiol with human endometrial and rat uterine estrogen receptors, and used a rat bioassay to assess their *in vivo* uterotropic activity.

MATERIALS AND METHODS

Subjects

Five healthy postmenopausal women aged 37–83 yr were studied. Subjects were in good health and were

taking no medication for at least 10 days before the experiment. For comparison and for standardization of the methods, the experiments were first carried out in two men aged 59–68 yr. All subjects had given informed consent before the procedure, and the project was approved by the Human Subjects Ethics Board of the Hospital and University.

Animals

The animals used were immature female Sprague–Dawley rats (18–19 days old) obtained from Charles River (Montreal). For estrogen receptor assays, the rats were decapitated and their uteri were removed, fat trimmed, flash frozen, and stored in liquid nitrogen. For some experiments frozen uteri processed as above were obtained from PEL–Freeze (Ar., USA).

Tissues

Endometrial samples were obtained from patients undergoing dilatation and curettage for diagnostic purposes and were processed as described previously [7].

Isotopes

[9,11-^{14}C] Δ^8-estrone ([^{14}C] Δ^8-E_1; 65 mCi/mmol) was obtained through custom synthesis (Amersham), and was provided by Wyeth–Ayerst (Philadelphia). It was purified by HPLC using system A. The major peak at 25 min corresponded in mobility to authentic unlabeled Δ^8-E_1 (Wyeth–Ayerst, Philadelphia). This material was repurified by using system B and its radiochemical purity was found to be over 95%. The purity was further confirmed by conversion to [^{14}C] Δ^8E_1-3-acetate. A single peak of radioactive material corresponding to authentic Δ^8-E_1-3-acetate, was observed. Prior to each experiment, the radiochemical purity of [^{14}C] Δ^8-E_1 was rechecked by HPLC. [2,4,6,7-^3H]-17β-estradiol ([^3H]-17β-E_2; 100 Ci/mmol) was obtained from NEN (Dupont, Boston, MA) and was over 95% pure when checked by HPLC using system B (retention time 18 min).

Measurement of radioactive materials

Radioactivity was measured in a liquid scintillation spectrometer (Beckman model LS 5000 TA, Toronto, Ontario). The radioactive materials in aqueous samples were measured by counting 0.5 ml aliquots in 3.5–5 ml of scintillation cocktail (Ready Value, Beckman). All other samples were counted using 5 ml toluene phosphor containing 0.4% Omnifluor (Scintillation dry mix, BDH, Toronto). Each sample was counted for sufficient time to bring the counting errors to less than 2%. All counts were corrected to 100% efficiency by external standardization.

Extraction of plasma

Plasma was extracted as described previously [3], and the method summarized in Fig. 2. Briefly, to monitor procedural losses, known amounts (0.2 μmol; 50 μg) of carrier Δ^8-E_1 and Δ^8-17β-E_2 were added to each plasma sample. The plasma (10–12 ml) was then fractionated into unconjugated, sulfate and glucuronide fractions. The glucuronide fraction contained insufficient amount of radioactivity and was not processed further. Residues from extracts containing unconjugated and sulfate conjugated estrogens were stored at –20°C until further processing.

Purification of steroids

Details of methods used to purify the various estrogens have been previously described [3, 4, 8]. Briefly, steroids were purified by reverse phase high performance liquid chromatography (HPLC) using a Beckman system Gold model 126 equipped with a DU 167 UV detector and a ODS-C18 column (4.6 x 250 mm or 10 x 250 mm). The solvent systems used were (A) acetonitrile–water–acetic acid (37:63:1); (B) acetonitrile–water–acetic acid (35:65:1); (C) acetonitrile–water–acetic acid (50:50:1). During the development of the purification procedure, ether extracts containing estrogens originally present in the sulfate conjugated form or the unconjugated form were subjected to HPLC using system A (flow rate 4.5 ml/min). Fractions containing Δ^8-E_1 (retention time 25–26 min) and Δ^8-17β-E_2 (retention 15.5 min) were pooled separately, and each estrogen was re-purified by a second HPLC using system B. In each instance, a single UV (280 nm)-absorbing material corresponding in mobility to Δ^8-E_1 or Δ^8-17β-E_2 was observed. Fractions containing each estrogen were pooled and evaporated to dryness. The residues were dissolved in methanol, aliquots were taken for determination of the amount of radioactivity and the mass of carrier steroid remaining after the above purification. These developmental experiments also indicated that a single HPLC purification step (system A) was sufficient to obtain radiochemical purity (>95%). This was confirmed by first conversion of the Δ^8-E_1 to Δ^8-E_1-3 acetate and Δ^8-17β-E_2 to Δ^8-8;17β-E_2-3 methyl ether followed by HPLC using system C. With both estrogen derivatives, a single peak of radioactivity and UV absorbing material corresponding in mobility to Δ^8-E_1-3 acetate (retention time 24 min) or Δ^8-17β-E_2-3-methyl ether (retention time 23 min) was observed. Therefore, in all subsequent experiments, a single HPLC purification step using system A, was used.

Determination of procedural losses

The mass of carrier Δ^8-estrone and Δ^8-17β-E_2 remaining after purification were determined by measuring the optical density at 280 nm using a

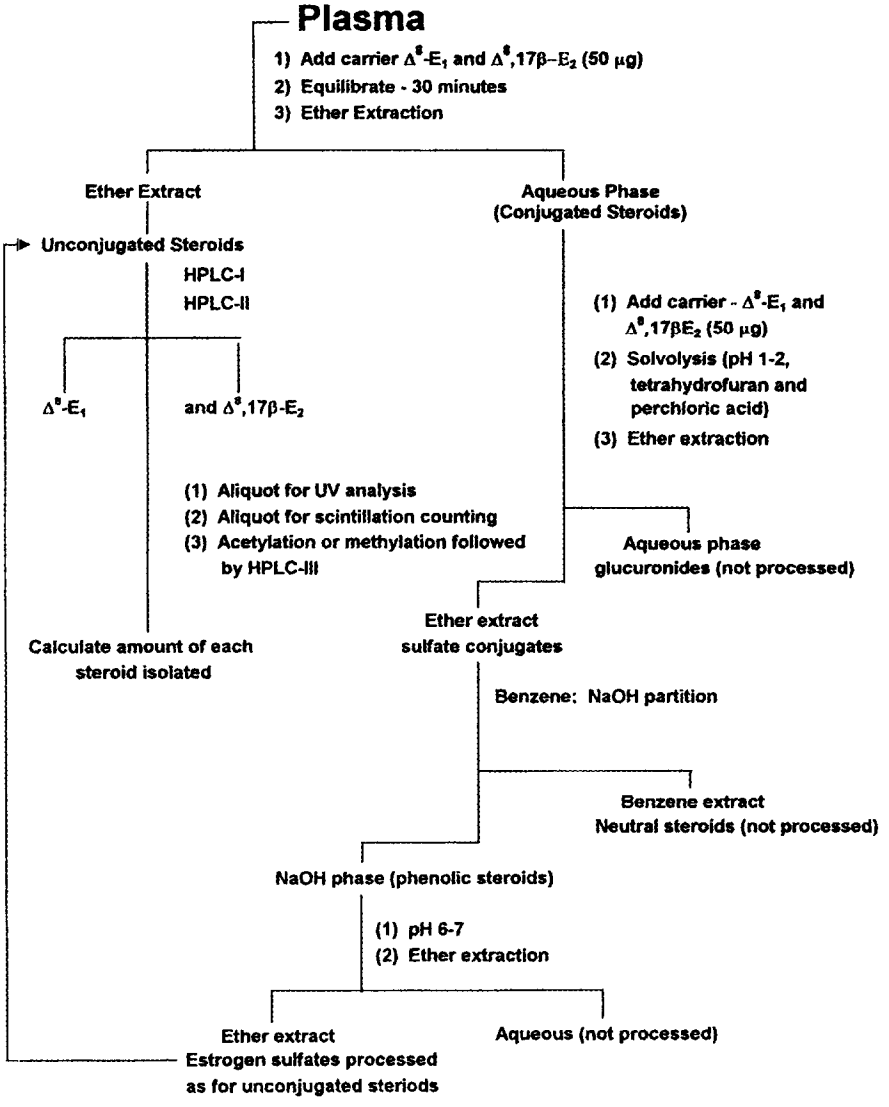

Fig. 2. Scheme for the extraction of steroids from the plasma.

Beckman model 65 DU spectrophotometer. The percent recovery was calculated and these data were used to correct the amount of radioactive estrogen isolated for procedural losses.

Pulse injection studies

These studies were carried out as described previously [3]. All experiments were started between 8 and 10 a.m. Subjects received, in an anticubital vein, between 20–40 μCi of [^{14}C] Δ^8-E_1, dissolved in 10 ml of 10% ethanol in isotonic saline. Blood samples (20–25 ml) were drawn into heparinized tubes at various times from a vein in the opposite arm, and the plasma was stored at −20°C until analyzed. All samples from a single subject were processed at the same time.

Estrogen receptor assays

The receptor assays, using human endometrium and rat uterine cytosols, were performed essentially as described previously for other Premarin components [7]. Since high specific activity tritium labeled Δ^8-E_1 or Δ^8-17β-E_2 are not available, competitive inhibition assays were used to determine the apparent affinity constants. Briefly, in this assay, samples containing 0.2–5 nM of [^3H]-17β-E_2 were incubated in the absence or presence of an excess unlabeled Δ^8-E_1 (10 nM) or Δ^8-17β-E_2 (2 or 10 nM), and the bound and unbound fractions were separated using dextran-coated charcoal as described previously [7]. The affinity constant (K_a, M^{-1}) for [^3H]-17β-E_2 and its apparent affinity constant (K_a^1,

M^{-1}) in the presence of Δ^8-E_1 or Δ^8-17β-E_2 were calculated from competitive Scatchard plots or the Lineweaver–Burk plots as described previously [9, 10]. The affinity constants for Δ^8-E_1 and Δ^8-17β-E_2 (K_b, M^{-1}) were calculated from

$$K_a = K_a^1(1 + [B])K_b,$$

where B is the concentration of the competing steroid as described previously [11]. The relative binding affinities (RBA's) were also determined as described by Korenman [12]. In these assays, 2 nM [^3H]-17β-E_2 was incubated in the presence of various concentrations of unlabeled Δ^8-E_1 and Δ^8-17β-E_2. The concentration of the competing steroid required to reduce by 50% the specific binding of [^3H]-17β-E_2 to human endometrial or rat uterine preparations was used to calculate the RBA by

$$\text{RBA} = [I_{50}] - 17\beta - E_2/[I_{50}] - \text{competitor} \times 100,$$

as described previously [7].

Determination of uterotropic activity

Immature rats were injected intraperitoneally daily for 3 d with each of the ten components of Premarin and Δ^8-17β-E_2 in their unconjugated form (2 μg of each estrogen in 100 μl of ethanol:saline, 1:9). The rats were euthanized 24 h after the last injection, and the uteri were excised, freed of fat, and weighed. The increase in uterine weight expressed as the ratio of mean uterine weight to mean body weight was taken as a measure of uterotropic activity. In these studies, a 2 μg dose was selected based on previous reports [13] that maximal uterotropic response was attained with 2 μg 17β-E_2 given daily for 3 d.

Analysis of pharmacokinetic data

The metabolic clearance rate (MCR) is defined as the volume of plasma irreversibly cleared of its hormone content per unit time (l per d or l per d/m^2). The data for the disappearance of radioactivity as Δ^8-E_1 indicated that the pattern could be represented by at least two exponentials, and the MCR of Δ^8-E_1 was calculated using the two compartment model described by Tait and Burstein [6] from the following relationship: $\text{MCR} = (\alpha \times \beta)/(A^1\beta + B^1\alpha)$, where A^1 and B^1 are the intercepts on the ordinate axis obtained by extrapolation of the two curves to zero time [3], α is the slope of the first (fast) component, and β is the slope of the second (slow) component of the curve. The initial volume of distribution was calculated from the relationship $V_1 = 1/(A^1 + B^1)$ and represents the volume in which the intravenously administered hormone was immediately distributed at time zero. The rate constant (k) of total removal (reversible and irreversible) of Δ^8-E_1 from the inner volume V_1 was calculated using the equation: $k = A^1\alpha + B^1\beta/(A^1 + B^1)$, as described previously [3, 14] The percentage of Δ-E_1 removed irreversibly was calculated from the relationship MCR/kV_1.

RESULTS

Pharmacokinetics of Δ^8-estrone

After i.v. administration of [^{14}C] Δ^8-8-E_1 to two men and five postmenopausal women, blood samples were drawn at various time intervals for up to 8 h. From the plasma, unconjugated Δ^8-E_1 was isolated, purified by HPLC, and the concentration of radioactivity as Δ^8-E_1, expressed as a percentage of the administered dose was plotted against time. The disappearance of radioactivity as Δ^8-E_1 in each subject could be expressed as a function that is the sum of two exponentials. Examples of the plots from subject 3 and 4 are shown in Fig. 3. The data obtained from the analysis of each individual disappearance curve, are given in Table 1. The mean value of the initial volume of distribution (V_1) was 19.8 $\pm$ 2 l, and the disappearance of Δ^8-E_1 from this inner pool had a mean half-life ($t_{1/2}$) of 5 $\pm$ 0.2 min. The mean $t_{1/2}$ of the slower component of Δ^8-E_1 was 40.4 $\pm$ 4 min (Table 1). The mean rate constant k of total removal of Δ^8-E_1 from V_1 was 232 $\pm$ 16 U/d (Table 1). The mean fraction of Δ^8-E_1 removed irreversibly (MCR/kV_1) was 0.65 $\pm$ 0.04. The mean (all subjects) MCR was 3035 $\pm$ 380 l/d or 1711 $\pm$ 252 l/d m^2. The mean MCR in postmenopausal women was 3570 $\pm$ 150 l/d or 2050 $\pm$ 147 l/d m^2. The mean MCR in the two men was 1697 $\pm$ 440 l/d or 848 $\pm$ 220 l/d m^2. These

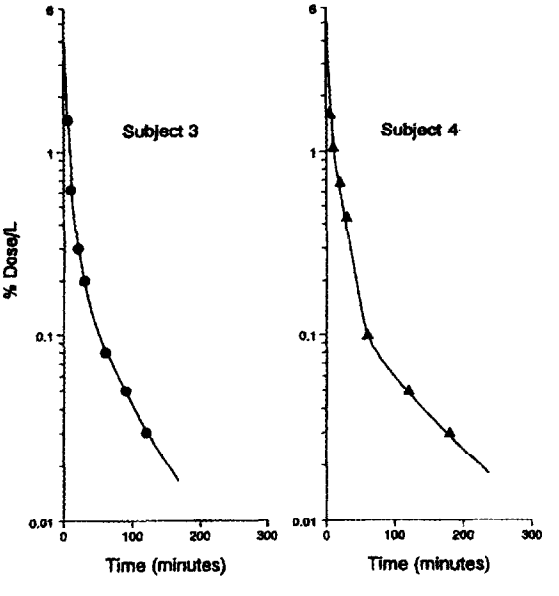

Fig. 3. Disappearance of radioactivity from plasma as [^{14}C]Δ^8-E_1 plotted as a percentage of the administered dose vs time of blood sampling in (subjects 3 and 4).

Table 1. Data calculated from analysis of Δ^8-estrone plasma concentration curves

Subject No.	Age (yrs)–sex	(^{14}C)Δ^8-E1 (dpm × 10^6)	Fraction of dose A′	Fraction of dose B′	α (U/day)	β (U/day)	k (U/day)	V_1 (l)	$t_{1/2}$ (min) components 1	$t_{1/2}$ (min) components 2	MCR (l/day)	MCR (l/day m^2)	MCR/kV_1
1	59 (M)	58.0	0.038	0.0062	220	21.0	189	22.0	6	50	2133	1066	0.50
2	66 (M)	38.6	0.110	0.0025	188	11.0	184	9.0	6	60	1261	630	0.76
3	47 (F)	59.6	0.036	0.0045	243	34.0	219	25.0	5	35	3580	1990	0.65
4	83 (F)	52.0	0.038	0.0025	241	19.0	227	25.0	5	40	3458	2034	0.60
5	70 (F)	49.0	0.047	0.0028	255	30.0	242	20.0	5	35	3595	2055	0.74
6	37 (F)	41.0	0.044	0.004	328	36.0	303	21.0	5	28	4069	2543	0.59
7	67 (F)	27.0	0.060	0.0042	280	36.0	263	17.0	5	35	3150	1660	0.72
	Mean ± SEM		0.05 ± 0.01	0.0004 ± 0.0005	250 ± 17.0	26.7 ± 37.0	232 ± 16.0	19.8 ± 2.0	5 ± 0.2	40.4 ± 4.0	3035 ± 380.0	1711 ± 252.0	0.65 ± 0.04

Table 2. Plasma levels of [^{14}C]-labeled unconjugated and sulfate conjugated metabolites formed after a bolus injection of [^{14}C]Δ^8-estrone to five postmenopausal women and two men

Metabolite isolated	Plasma levels (% dose/l; mean ± SEM) 5 min[a]	10 min	20 min	30 min	60 min	90 min[b]	120 min	180 min	240 min	360 min	480 min
Δ^6-17β-E_2	0.55 ± 0.10	0.60 ± 0.14	0.43 ± 0.07	0.30 ± 0.05	0.18 ± 0.02	0.13 ± 0.01	0.10 ± 0.01	–	–	–	–
Δ^8-E_1S	0.36 ± 0.14	0.45 ± 0.06	0.68 ± 0.10	0.72 ± 0.10	0.86 ± 0.12	1.00 ± 0.20	0.76 ± 0.14	0.68 ± 0.16	0.60 ± 0.14	0.52 ± 0.14	0.40 ± 0.09
Δ^8-17β-E_2S	0.35 ± 0.09	0.47 ± 0.10	0.70 ± 0.14	0.74 ± 0.14	0.93 ± 0.16	0.90 ± 0.13	0.87 ± 0.10	0.70 ± 0.10	0.64 ± 0.10	0.60 ± 0.10	0.46 ± 0.10

[a]Blood samples obtained from only four subjects.
[b]Blood samples obtained from only three subjects.

results indicate that the MCR in the men appears to be approx. $2\frac{1}{2}$ times lower than in women.

Formation and kinetics of unconjugated Δ^8-17β-estradiol after a bolus injection of [^{14}C] Δ^8-estrone

From the plasma unconjugated fraction (Fig. 2), the only major metabolite isolated and identified was [^{14}C] Δ^8-17β-E_2. The amounts formed after injection of [^{14}C] Δ^8-E_1 were measured in two men and five postmenopausal women. The mean ± SEM values are summarized in Table 2. Δ^8-17β-E_2 was formed very rapidly, with the maximum concentration present in the 10 min sample (Fig. 4). The levels then gradually declined in a manner similar to the disappearance curve of the parent steroid [^{14}C] Δ^8-E_1 (Fig. 3). After 2 h, [^{14}C] Δ^8-17β-E_2 was barely detectable. The bulk of the radioactive material present in the unconjugated fraction could be accounted for by the amounts present in Δ^8-E_1 and Δ^8-17β-E_2.

Formation and kinetics of Δ^8-estrone sulfate and Δ^8-17β-estradiol sulfate after a bolus injection of [^{14}C] Δ^8-estrone

From the plasma sulfate conjugated fraction, [^{14}C]-labeled Δ^8-17β-E_2S and Δ^8-E_1S were isolated, purified, and the amounts formed were measured in two men and five postmenopausal women after injection of [^{14}C] Δ^8-E_1. The appearance of radioactivity in these two metabolites of [^{14}C] Δ^8-E_1, expressed as a percentage of the dose per l (mean levels of 7 subjects), at various time intervals, are summarized in Table 2. Both Δ^8-E_1S and Δ^8-17β-E_2S (Fig. 5) appeared in the plasma gradually and reached maximum levels between 60–90 min. Almost equal amounts of Δ^8-E_1S and Δ^8-17β-E_2S were formed over the 8 h blood sampling period (Table 2). The disappearance of both Δ^8-E_1S and Δ^8-17β-E_2S

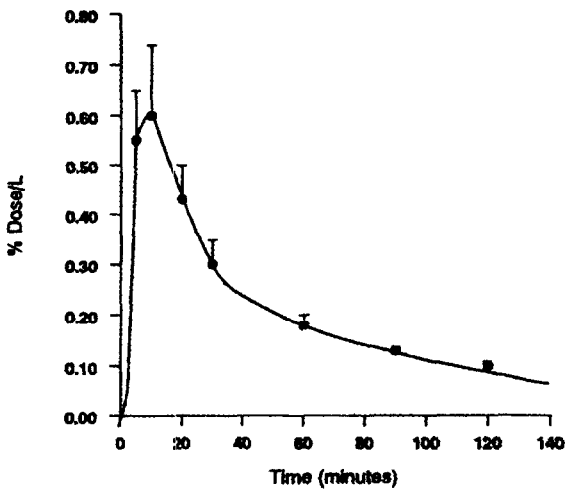

Fig. 4. Kinetics of [^{14}C]Δ^8-17β-E_2 formation after i.v. administration of [^{14}C]Δ^8-E_1. Values shown are the mean ± SEM (subjects 1–7).

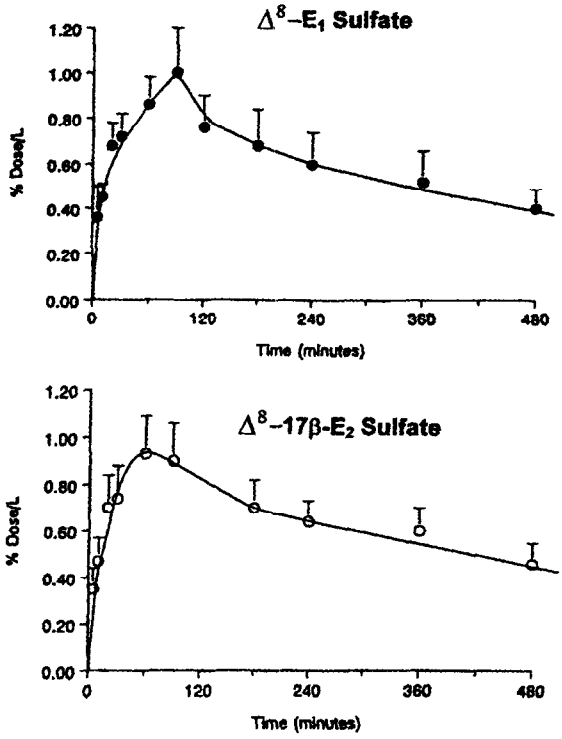

Fig. 5. Kinetics of [^{14}C]Δ^8-E_1S and [^{14}C]Δ^8-17β-E_2S formation after i.v. administration of [^{14}C]Δ^8-E_1. Values shown are the mean ± SEM (subjects 1–7).

(Fig. 5) were characterized by two components, $t_{1/2}$ of the first component being approx. 5 and 4 h, respectively. The $t_{1/2}$ of the second component of the Δ^8-E_1 and Δ^8-17β-E_2S and Δ^8-E_1S elimination curves were approx. 12 and 15 h, respectively. As observed in the plasma unconjugated fraction, the bulk of the radioactive material present in the plasma sulfate conjugated form could also be accounted for by the amounts present in the two sulfate metabolites isolated. No labeled equilin sulfate, estrone sulfate or 17β-estradiol sulfate were detectable. These results indicate that Δ^8-E_1 is not a precursor for Δ-7-ring B unsaturated estrogens (Eq,17β-Eq) or the B ring saturated estrogens (E_1,17β-E_2).

Binding of Δ^8-estrone and Δ^8-17β-estradiol to human endometrial and rat uterine cytosol receptors

The relative binding affinities K_b, (K_a) of Δ^8-E_1, and Δ^8-17β-E_2 for human endometrium and rat uterine cytosol receptors were determined by competitive Scatchard analysis as described in Section 2. In one such experiment, different concentrations of [^3H]-17β-E_2 were incubated in the presence of 2 or 10 nM unlabeled competitor; the results are shown in Figs 6 and 7. The data indicate that both Δ^8-E_1 and Δ^8-17β-E_2 can effectively compete with 17β-E_2 for the binding sites. The data further indicate that the

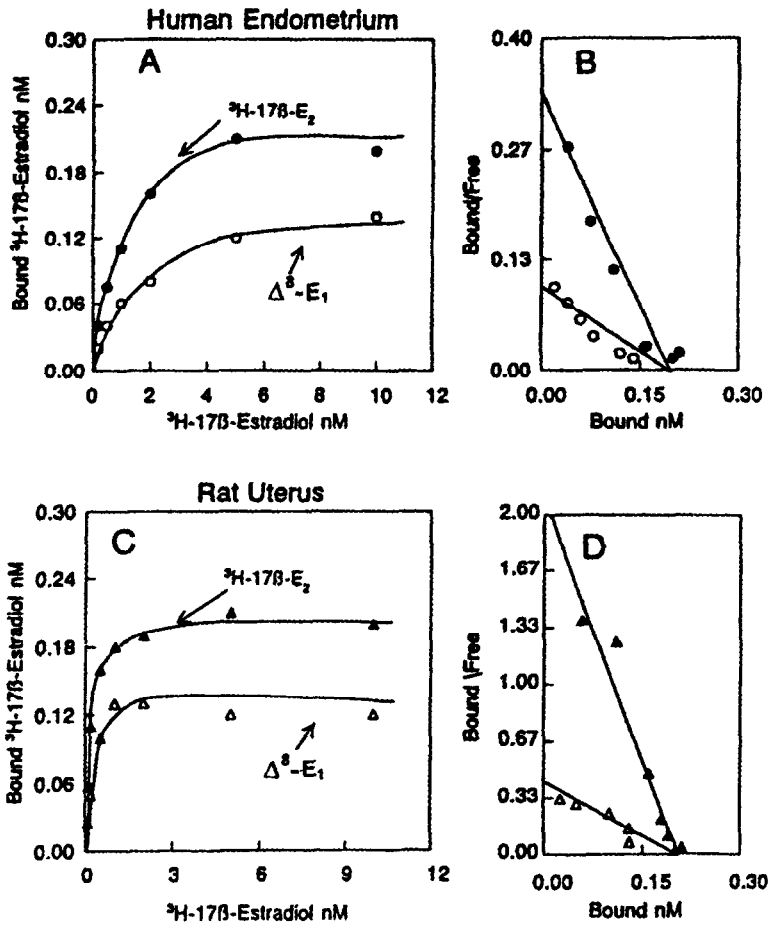

Fig. 6. Specific binding of [^3H]-17β-E_2 with human endometrial (A and B) and rat uterine (C and D) cytosols in the presence of 10 nM unlabeled Δ^8-E_1. These data were used for the calculation of the affinity constants from Scatchard plots (B and D), as described in Section 2.

Scatchard plots are linear and indicate the presence of a single class of high affinity binding sites. The apparent affinity constants ($K_b \times 10^9\ M^{-1}$) for Δ^8-E_1 and Δ^8-17β-E_2 for human endometrial receptors were 0.3, and $1 \times 10^9\ M^{-1}$, respectively. The corresponding values for rat uterine receptors were 0.45 and $2 \times 10^9\ M^{-1}$, respectively. The corresponding disassociation constants (K_d) were 3.6, 1, 2.2 and 0.5×10^{-9} M, respectively. The affinity constants for 17β-E_2 were 2 and $10 \times 10^{-9}\ M^{-1}$ for human endometrial and rat cytosol receptors, respectively (Figs 6 and 7). Thus, compared to 17β-E_2, the relative affinity constants for Δ^8-E_1 and Δ^8-17β-E_2 for human endometrial and rat cytosol receptors are approx. 7, 22, 2 and 5-fold lower, respectively.

The RBAs of Δ^8-E_1 and Δ^8-17β-E_2 for cytosol receptors were also determined by a competitive binding assay using 2 nM [^3H]-17β-E_2 as the labeled ligand and increasing concentrations of unlabeled Δ^8-E_1 and Δ^8-17β-E_2. The results indicate (Fig. 8), that the typical 'S' shaped binding curves are essentially parallel, and that the relative potency estimates can be determined by calculating the amount of Δ^8-E_1 and Δ^8-17β-E_2 required to reduce the [^3H]-17β-E_2-specific binding by 50%. The parallel nature of displacement curves also indicates that the binding is competitive. For Δ^8-E_1, approx. 95–100 and 190–200 nM were required to reduce the [^3H]-17β-E_2 binding by 50% for the human endometrial and rat uterine receptors, respectively. Similarly, approx. 23–25 and 7–8 nM Δ^8-17β-E_2 were required to reduce the [^3H]-17β-E_2 binding for human endometrial and rat uterine receptors, respectively. Thus the relative RBA's calculated for Δ^8-E_1 and Δ^8-17β-E_2 for human endometrial and rat uterine receptors are 0.8%, 3%, 0.4% and 11% that of 17β-E_2, respectively. These results also clearly indicate that in both species, Δ^8-17β-E_2 has a much greater affinity for estradiol receptors than Δ^8-E_1. Thus in the human, it is approx. 4 times and in the rat 25 times more active than Δ^8-E_1.

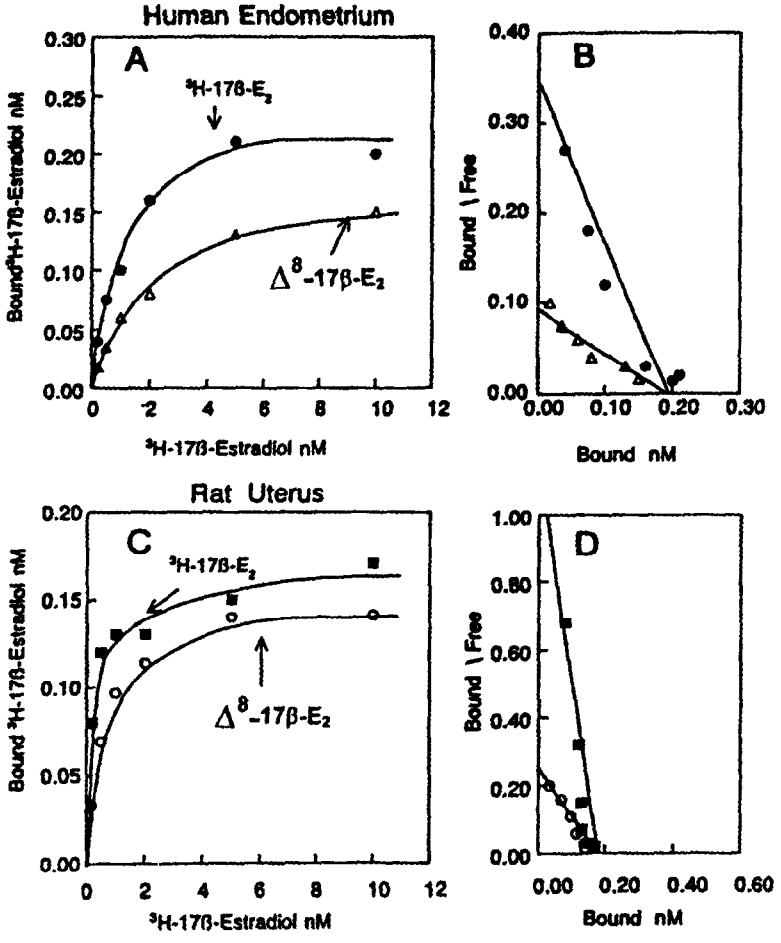

Fig. 7. Specific binding of [^3H]-17β-E_2 with human endometrial (A and B) and rat uterine (C and D) cytosols in the presence of 2 nM unlabeled Δ^8-17β-E_2. These data were used for the calculation of the affinity constants from Scatchard plots (B and D) as described in Section 2.

Similar results were also observed with rat uterine nuclear preparations (data not shown).

Uterotropic activities of Δ^8-E_1 and Δ^8-17β-E_2

In these experiments, a single dose (2 μg/100 g body weight/d) of each of the ten individual components of Premarin and Δ^8-17β-E_2 the major *in vivo* metabolite of Δ^8-E_1 in man was administered i.p. to immature rats for 3 d. The uterine weight was determined 24 h after the last injection. The results are shown in Table 3. In this bioassay, except for the 17α-E_2, all other estrogens brought about a significant ($p < 0.05$) increase in uterine weight compared to the controls. The most active estrogens were Eq, E_1 and their corresponding 17β-reduced derivatives 17β-Eq and 17β-E_2, respectively. Both Δ^8-E_1 and its 17β-reduced derivative Δ^8-17β-E_2 were active uterotropic agents, though somewhat less potent. The order of uterotropic potency based on a single dose (2 μg), given for 3 d, was Eq $\geq$ 17β-Eq $\geq$ $E_1 \geq$ 17β-E_2 > Δ^8-17β-E_2 > Δ^8-E_1 > Eqn $\geq$ 17β-Eqn > 17α-Eqn $\geq$ 17α-Eq > 17α-E_2 > control.

DISCUSSION

Δ^8-E_1S is the most recent component identified in Premarin. Approx. 3.5% of the total estrogens present in Premarin consists of Δ^8-E_1S. This estrogen in its radioactive form was not suitable for pharmacokinetic studies in humans. However, previous studies with other estrogens such as EqS, 17β-EqS and E_1S, indicate that, after administration of these conjugated estrogens to postmenopausal women and men, unconjugated Eq, 17β-Eq and E_1 are readily found in the circulation [3, 4, 15, 16]. It is also generally accepted that it is the unconjugated estrogen that is biologically active. Therefore, in the present study, the pharmacokinetics of unconjugated Δ^8-E_1 and its *in vivo* metabolism were investigated in both postmenopausal women and men. The mean MCR of Δ^8-E_1

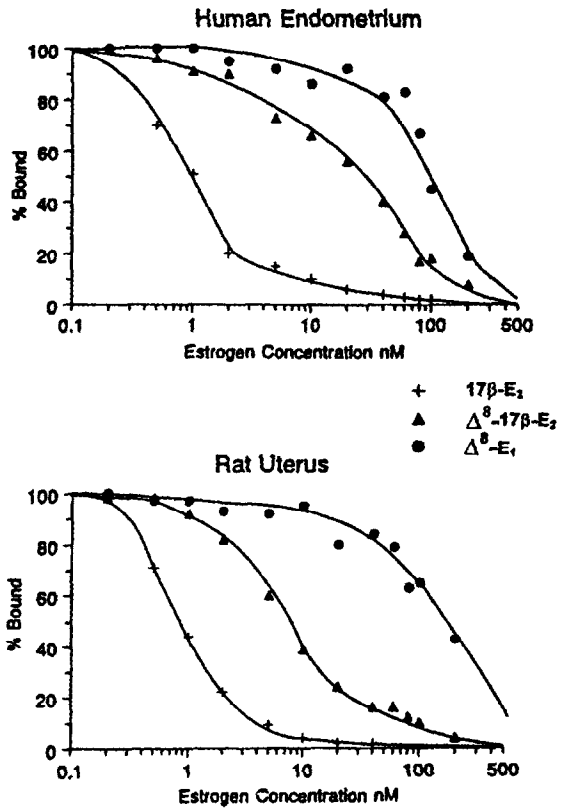

Fig. 8. Competitive binding assays of Δ^8-E_1 and Δ^8-17β-E_2 with human endometrial and rat uterine cytosol receptors. Cytosols were incubated with 2 nM [^3H]-17β-E_2 and various concentrations of unlabeled competitors, as described in Section 2.

was calculated by analysis of plasma samples taken after a bolus injection of [^{14}C] Δ^8-E_1. The data points (Fig. 3) showing the elimination of Δ^8-E_1 from plasma, suggest that a two-compartment model can be used for the analysis of the MCR of this estrogen, as described previously for 17β-Eq [4]. In contrast, the disappearance of equilin from plasma indicated that a single compartment model was sufficient to describe its elimination [3].

The mean MCR of Δ^8-E_1 was 1711 ± 252 l/d m^2. The $2\frac{1}{2}$-fold difference between the two men and the five postmenopausal women, and the differences between subjects are most likely due to normal individual variations in fractional turnover rates, volumes of distribution, and hepatic and renal blood flow.

Table 3. Rat uterine responses to intraperitoneal administered equine estrogens

Estrogen	No. of rats	Uterine weight (mg) mean ± SEM	Uterine weight per body weight (mg/g) ± SEM
Equilin	20	98.8 ± 5.0[a]	2.30 ± 0.13[a]
17β-Dihydroequilin	20	98.6 ± 6.0[a]	2.30 ± 0.15[a]
17β-Estradiol	20	89.4 ± 4.6[a]	2.20 ± 0.10[a]
Estrone	20	88.4 ± 8.0[a]	2.10 ± 0.16[a]
Δ^8-17β-Estradiol	15	75.0 ± 3.0[a]	1.70 ± 0.10[a]
Δ^8-Estrone	20	60.5 ± 2.0[a]	1.38 ± 0.04[a]
Equilenin	20	52.6 ± 2.0[a]	1.40 ± 0.05[a]
17β-Dihydroequilenin	20	51.9 ± 2.0[a]	1.20 ± 0.05[a]
17α-Dihydroequilenin	20	39.0 ± 2.0[a]	0.90 ± 0.04[a]
17α-Dihydroequilin	20	38.5 ± 1.0[a]	0.90 ± 0.02[a]
17α-Estradiol	20	37.4 ± 2.0[b]	0.95 ± 0.04[b]
Control	30	33.8 ± 1.0	0.77 ± 0.02

[a]Significantly different from control $P < 0.01$.
[b]Not significant.

Similar differences in individual MCR's have been observed with other estrogens and steroids [3, 15–17]. The mean MCR of Δ^8-E_1 is approx. 1.5 times lower than that of Eq 2641 l/d m^2 [3], but is higher than that of 17β-Eq, 1252 l/d m^2 [4] and 17β-E_2, 580–820 l/d m^2 [16, 18, 19] and similar to that of E_1, 965–1400 l/d m^2 [16, 19]. These observations and the data from the present study indicate that unconjugated Δ^8-E_1 can circulate in the body longer than Eq, but compared to the biologically more potent 17β-reduced estrogens, it is cleared more rapidly.

The V_1 (19.8 $\pm$ 2.0 l) for Δ^8-E_1 is much larger than plasma volume, and this would suggest relatively low binding affinity of Δ^8-E_1 for serum binding proteins such as sex hormone binding globulin (SHBG) and albumin. The disappearance of Δ^8-E_1 from this inner volume of distribution had a mean half-life of 5 $\pm$ 0.2 min and this is similar to that reported for 17β-Eq [4]. The mean rate constant (k) of total removal (reversible plus irreversible) of Δ^8-E_1 from the circulation was 232 $\pm$ 16 U/d, and the ratio of irreversible over total removal (MCR/kV_1; Table 1) had a mean value of 0.65 $\pm$ 0.04, indicating that 65% of Δ^8-E_1 is lost from the circulation irreversibly. These values are in keeping with the high MCR of Δ^8-E_1.

In the present study, the formation of other conjugated and unconjugated metabolites of Δ^8-E_1 was also investigated. From the unconjugated fraction, the only other major radioactive metabolite present and identified was Δ^3-17β-E_2 (Table 2, Fig. 4). The bulk (>90%) of the radioactivity present in the unconjugated fraction, was associated with the parent compound Δ^8-E_1 and its major 17β-reduced metabolite Δ^8-17β-E_2. Very small amounts of radioactivity remained unaccounted for. Similarly, in the sulfate conjugated form, the bulk of the radioactivity present could be accounted for in amounts present in Δ^8-E_1S and Δ^8-17β-E_2S. The rate of formation of these two metabolites indicates that maximal levels were attained between 60–90 min and that nearly equal amounts of the two metabolites were formed (Table 2, Fig. 5). In contrast, after administration of Eq (the main ring B unsaturated estrogen present in Premarin), a number of metabolites such as Eqn, 17β-Eq, and 17β-Eqn, were isolated from both the unconjugated and the sulfate conjugated fractions [3]. Moreover, the amounts of Eq metabolites formed, were much lower than the circulating levels of Eq and EqS [3]. Thus, it appears that the metabolism of Δ^8-E_1 is unique, in that the major *in vivo* metabolite in both the unconjugated and the sulfate conjugated form was the more active 17β-reduced product. These interconversions are depicted in Fig. 9.

We have previously reported [8] that under steady-state conditions, the transfer constants or ρ values [20–22], for the conversion of EqS to Eq and 17β-Eq, were 0.25 and 0.15 respectively [3]. The corresponding values for the conversion of E_1S to E_1 and 17β-E_2 were 0.15–0.21 and 0.014–0.03, respectively [15, 16]. Since 17β-Eq and 17β-E_2 are the principal active metabolites of EqS and E_1S, respectively, the extent of this activation by 17β-reduction, is several fold higher for the ring B unsaturated estrogens. The data from the present study also support the above conclusions, in that the main metabolite of Δ^8-E_1 was the 17β-reduced compound. Furthermore, the amounts of Δ^8-E_1S and Δ^8-17β-E_2S found in the circulation following administration of Δ^8-E_1 were almost equal. This pattern of metabolism, i.e. equal amounts of 17-oxidized and 17β-reduced metabolites, has not been observed with other estrogens or even androgens. The oxidative pathway is considered to be the preferred one com-

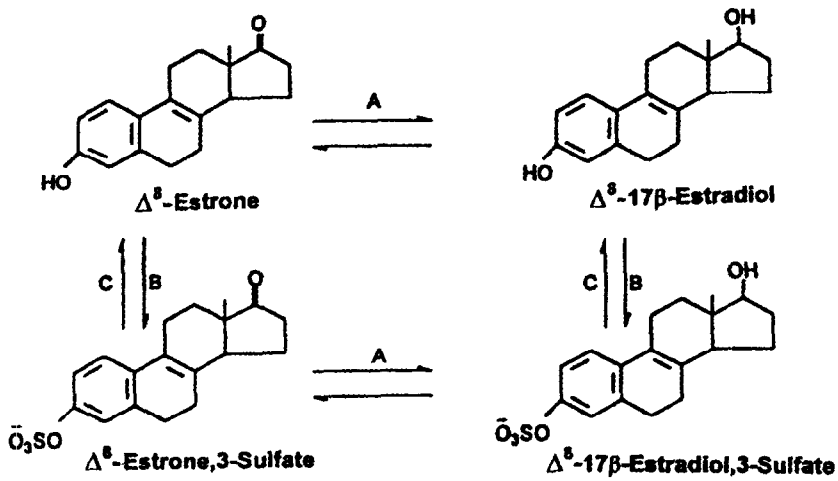

Fig. 9. Interconversion between Δ^8-E_1 and its unconjugated and sulfate conjugated metabolites in postmenopausal women and men (A = 17β-hydroxysteroid dehydrogenase; B = sulfokinase; C = sulfatase).

pared to the reductive pathway [22]. Though these results strongly suggest that the extent of activation by 17β-reduction of Δ^8-E_1 appears to be even greater with this ring B unsaturated estrogen, more quantitative experiments under steady-state conditions are needed before firm conclusions can be made.

The data from the present study also indicate that, unlike other ring B unsaturated estrogens such as Eq and 17β-Eq which are metabolized to a number of other (circulating) ring B unsaturated estrogens [3, 4, 8], Δ^8-E_1 was converted *in vivo* in both men and postmenopausal women to a single major metabolite Δ^8-17β-E_2 (unconjugated and sulfate conjugated). This lack of extensive metabolism may be due to the novel structure of Δ^8-E_1, i.e. the presence of a single conjugated double bond in the B ring. Whether Δ^8-E_1 and Δ^8-17β-E_2 are further metabolized prior to excretion in the urine remains to be investigated.

Both Δ^8-E_1 and its major metabolite, Δ^8-17β-E_2, can interact with human endometrial and rat uterine estrogen receptors in a manner similar to that described for other ring B unsaturated estrogens and 17β-estradiol [7]. Both of these estrogens bind with estrogen receptors with high affinity. The RBA of Δ^8-17β-E_2 was approx. 4 (human endometrium) and 25 (rat uterus) times higher than that of Δ^8-E_1. Similar results have been previously reported with other 17β-reduced estrogens [12]. Though the RBA of Δ^8-17β-E_2 is only 3 to 11% that of 17β-E_2 observed in the human endometrial and rat uterine preparations, respectively, yet this estrogen has substantial *in vivo* uterotropic activity as indicated by the bioassay data (Table 3). Whether this is due in part to the pharmacokinetics of Δ^8-E_1 and Δ^8-17β-E_2 or to the ability of various estrogens (with different binding affinities) to modulate distinct DNA response elements in a tissue selective manner as recently reported by Yang *et al.* [23] remains to be investigated. Along with the uterotropic activities of Δ^8-E_1 and Δ^8-17β-E_2, recent data [2] indicated that daily administration of small amounts (125 μg) of Δ^8-E_1S for 8 weeks to postmenopausal women significantly reduced the urinary levels of *N*-telopeptides of collagen type 1, a marker of bone resorption [24]. These results indicate that in the bone, Δ^8-E_1S acts like a potent estrogen. Moreover, in the above study, Δ^8-E_1S significantly delayed the oxidation of LDL cholesterol. Recent data also indicates that, among the several estrogens tested, Δ^8-E_1 and Δ^8-17β-E_2 were extremely active antioxidants [25]. This estrogenic effect does not appear to be mediated via the estrogen receptor but perhaps involves other mechanisms (non-genomic) but this remains to be determined.

In conclusion, the data presented demonstrate that Δ^8-E_1 has estrogenic activity and that it is metabolized in the human subject to a more potent estrogen: Δ^8-17β-E_2. The pharmacokinetic data indicate that Δ^8-E_1 and Δ^8-17β-E_2, circulate mainly as sulfates, and are slowly eliminated from the body. Both Δ^8-E_1 and Δ^8-17β-E_2 bind with estrogen receptors with high affinity and can therefore contribute to the overall *in vivo* biological effects of Premarin. These data further support previous conclusions that, in postmenopausal women, the biological effects of Premarin are the sum of the effects of its various individual components [1]. Therefore, conjugated estrogen preparations lacking some of these novel ring B unsaturated estrogens, will not provide the same extent of beneficial effects as Premarin.

Acknowledgements—This work was supported by the Medical Research Council of Canada grant MT-11929. We wish to thank Dr. Mike Dey, Wyeth–Ayerst, Philadelphia, U.S.A., for his generous gift of labeled and unlabeled Δ^8-E_1.

REFERENCES

1. Bhavnani B. R., The saga of the ring B unsaturated equine estrogens. *Endocrine Reviews* **9** (1988) 396–416.
2. Bhavnani B. R., Cecutti A. and Dey M. S., Effects in postmenopausal women of delta-8-estrone sulfate: A novel estrogen component of Premarin. *Journal Society Gynecologic Investigation* **4** (1 (Suppl.)) (1997) 392.
3. Bhavnani B. R., Woolever C. A., Benoit H. and Wong T., Pharmacokinetics of equilin and equilin sulfate in normal postmenopausal women and men. *Journal of Clinical Endocrinology and Metabolism* **56** (1983) 1048–1056.
4. Bhavnani B. R. and Cecutti A., Pharmacokinetics of 17β-dihydroequilin sulfate and 17β-dihydroequilin sulfate in normal postmenopausal women. *Journal Clinical Endocrinology and Metabolism* **78** (1994) 197–204.
5. Raveendranath, P. and Wichtowski, J. A., Alkali metal 8,9-dehydroestrone sulfate esters. U. S. Patent Feb. 22, 1994, 5, 288, 717.
6. Tait, J. F. and Burstein, S., *In vivo* studies of steroid dynamics in man. In *The Hormones*, ed. G. Pincus, K. V. Thimann and E. B. Astwood. Academic Press, New York, 1964, ch. 4, pp. 441–557.
7. Bhavnani B. R. and Woolever C. A., Interaction of ring B unsaturated estrogens with estrogen receptors of human endometrium and rat uterus. *Steroids* **56** (1991) 201–209
8. Bhavnani B. R. and Cecutti A., Metabolic clearance rate of equilin sulfate and its conversion to plasma equilin, conjugated and unconjugated equilenin, 17β-dihydroequilin, and 17β-dihydroequilenin in normal postmenopausal women and men under steady state conditions. *Journal Clinical Endocrinology and Metabolism* **77** (1993) 1269–1274.
9. Scatchard G., The attraction of proteins for small molecules and ions. *Annals New York Academy of Science* **51** (1949) 660–672.
10. Pan C. C., Woolever C. A. and Bhavnani B. R., Transport of equine estrogens: binding of conjugated and unconjugated equine estrogens with human serum proteins. *Journal Clinical Endocrinology and Metabolism* **61** (1985) 499–509.
11. Edsall, J. T. and Wyman, J., *Biophysical Chemistry*. Academic Press, New York, 1958, pp. 591–662.
12. Korenman S. G., Comparative binding affinity of estrogens and its relation to estrogenic potency. *Steroids* **13** (1969) 163–177.
13. Katzenellenbogen B. S., Katzenellenbogen J. A. and Modecai D., Zaeralenones: Characterization of the estrogenic potencies and receptor interactions of a series of fungal β-resorcyclic acid lactones. *Endocrinology* **105** (1979) 33–40.
14. Rizkallah T., Gurpide E. and Vanderwiele R. L., Metabolism of HCG in man. *Journal Clinical Endocrinology* **29** (1969) 92–100.
15. Longcope C., The metabolism of estrone sulfate in normal males. *Journal Clinical Endocrinology and Metabolism* **34** (1972) 113–122.

16. Ruder H. J., Loriaux D. L. and Lipsett M. B., Estrone sulfate production rate and metabolism in man. *Journal Clinical Investigation* **51** (1972) 1020–1033.
17. Tait J. F., Tait S. A. S., Little B. and Laumas K. R., The disappearance of 7[^3H]aldosterone in the plasma of normal subjects. *Journal Clinical Investigation* **40** (1961) 72–80.
18. Longcope C., Metabolic clearance and blood production rates of estrogens in postmenopausal women. *American Journal Obstetrics and Gynecology* **111** (1971) 778–781.
19. Longcope C. and Tait J. F., Validity of metabolic clearance and interconversion rates of estrone and 17β-estradiol in normal adults. *Journal Clinical Endocrinology* **82** (1971) 481–490.
20. Gurpide E., MacDonald P. C., Vande Wiele R. L. and Lieberman S., Measurement of the rates of secretion and of peripheral metabolism of two interconvertible compounds: dihydroisoandrosterone–dehydroisoandrosterone sulfate. *Journal Clinical Endocrinology and Metabolism* **23** (1968) 346–354.
21. Horton R. and Tait J. F., Androstenedione production and interconversion rates measured in peripheral blood and studies on the possible site of its conversion to testosterone. *Journal Clinical Investigation* **45** (1966) 301–313.
22. Baird D. T., Horton R., Longcope C. and Tait J. F., Steroid dynamics under steady-state conditions. *Recent Progress Hormone Research* **25** (1969) 611–664.
23. Yang Na. N., Venugopalan M., Hardikor S. and Glasebrook A., Identification of an estrogen response element activated by metabolites of 17β-estradiol and raloxifene. *Science* **273** (1996) 1222–1225.
24. Calvo M. S., Eyre D. R. and Grundborg C. M., Molecular basis and clinical application of biological markers of bone turnover. *Endocrine Reviews* **17** (1996) 333–368.
25. Bhavnani B. R., Cecutti A., Gerulath A. and Woolever C. A., Are cardiovascular benefits associated with conjugated equine estrogen therapy and red wine consumption-A result of the antioxidant properties of their components. *Journal Society Gynecologic Society* **4** (1) (1997) 106A.

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

BHAGU R. BHAVNANI[1]

Department of Obstetrics and Gynecology and Institute of Medical Sciences and St. Michael's Hospital, University of Toronto, Toronto, Ontario, Canada M5B 1W8

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

[1] To whom requests for reprints should be addressed at Department of Obstetrics and Gynecology, St. Michael's Hospital, 30 Bond Street, Toronto, Ontario M5B 1W8.
This work was supported by the Medical Research Council of Canada, Grant MT-3724 and MT-11929.

0037-9727/98/2171-0006$10.50/0

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

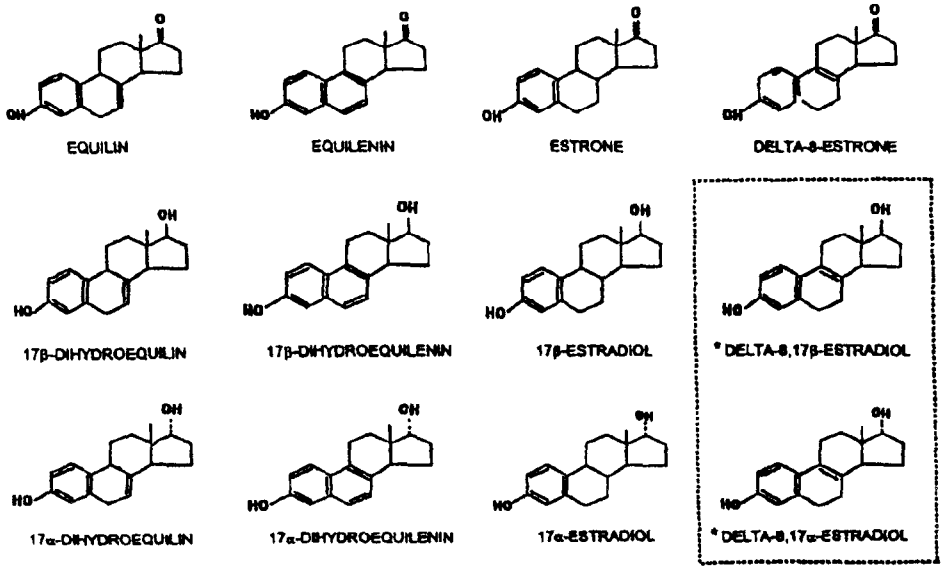

Figure 1. Some of the estrogens isolated from the urine of pregnant mares. These estrogens in their sulfate ester form are present in the drug Premarin.

*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 $^3H/^{35}S$ ratio

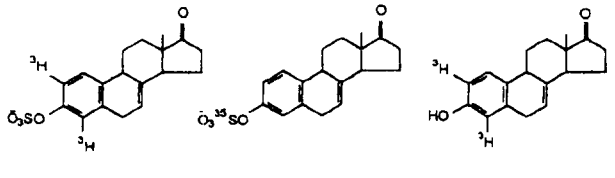

Figure 2. Labeled equilin sulfate and equilin used in absorption studies (Experiment. A and B—1:1 mixture of I and II was used; Experiment C—1:2 mixture of II and III was used.

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, $^3H/^{35}S$ = 1) or [^{35}S]Equilin and Equilin [^{35}S]Sulfate (Expt. C, $^3H/^{35}S$ = 2) to Normal Men

Time (min)	A $^3H/^{35}S$	B $^3H/^{35}S$	C $^3H/^{35}S$
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 ($^3H/^{35}S$ = 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

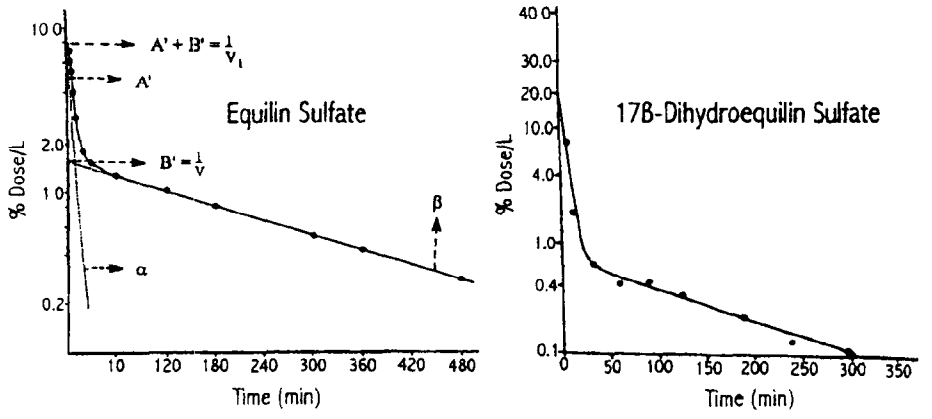

Figure 3. Disappearance of radioactivity from plasma as [^{3}H]equilin sulfate and [^{3}H]17β-dihydroequilin sulfate plotted as a percentage of the administered dose versus time of blood sampling. (Adapted with permission from Ref. 25 and 26.)

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)	$t_{½}$ 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.

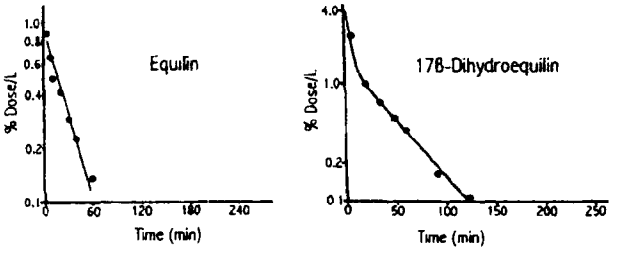

Figure 4. Disappearance of radioactivity from plasma as [^{3}H]equilin and [^{3}H]17β-dihydroequilin plotted as a percentage of the administered dose versus time of blood sampling. (Adapted with permission from Ref. 26.)

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

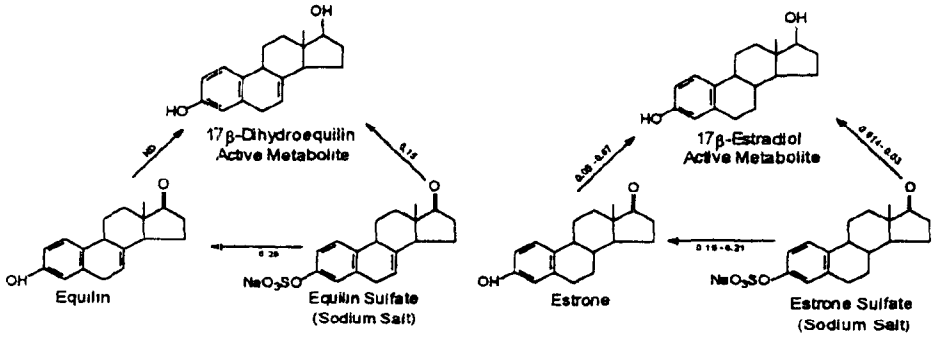

Figure 5. Extent of *in vivo* activation of the two main components of conjugated equine estrogen preparations by 17β-hydroxysteroid dehydrogenase. The values shown are transfer constants.

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)	$t_{1/2}$ components (minutes)		MCR (L/day)	MCR (L/day.m²)	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

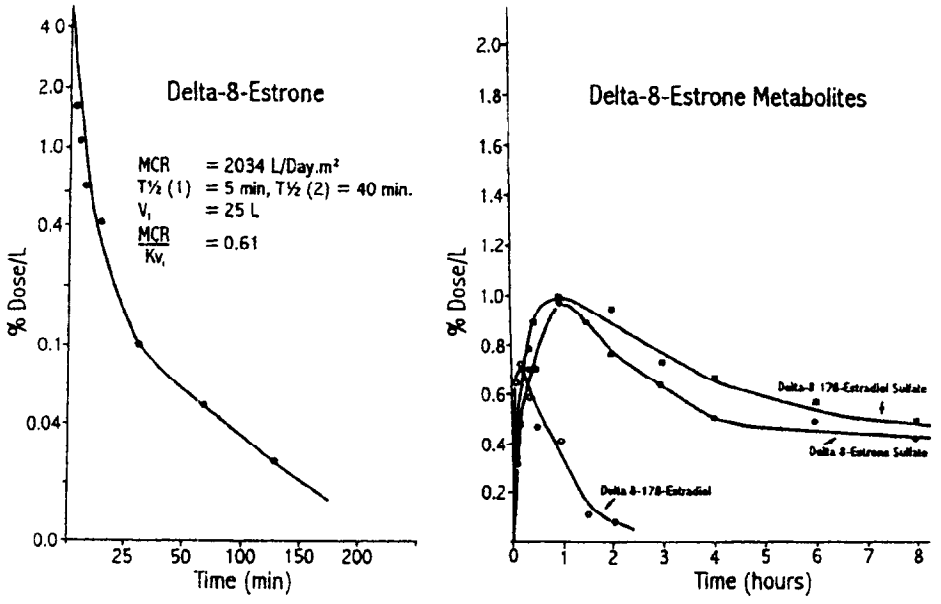

Figure 6. Pharmacokinetics of delta-8-estrone and its three metabolites, after administration of [^{14}C]delta-8-estrone.

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.

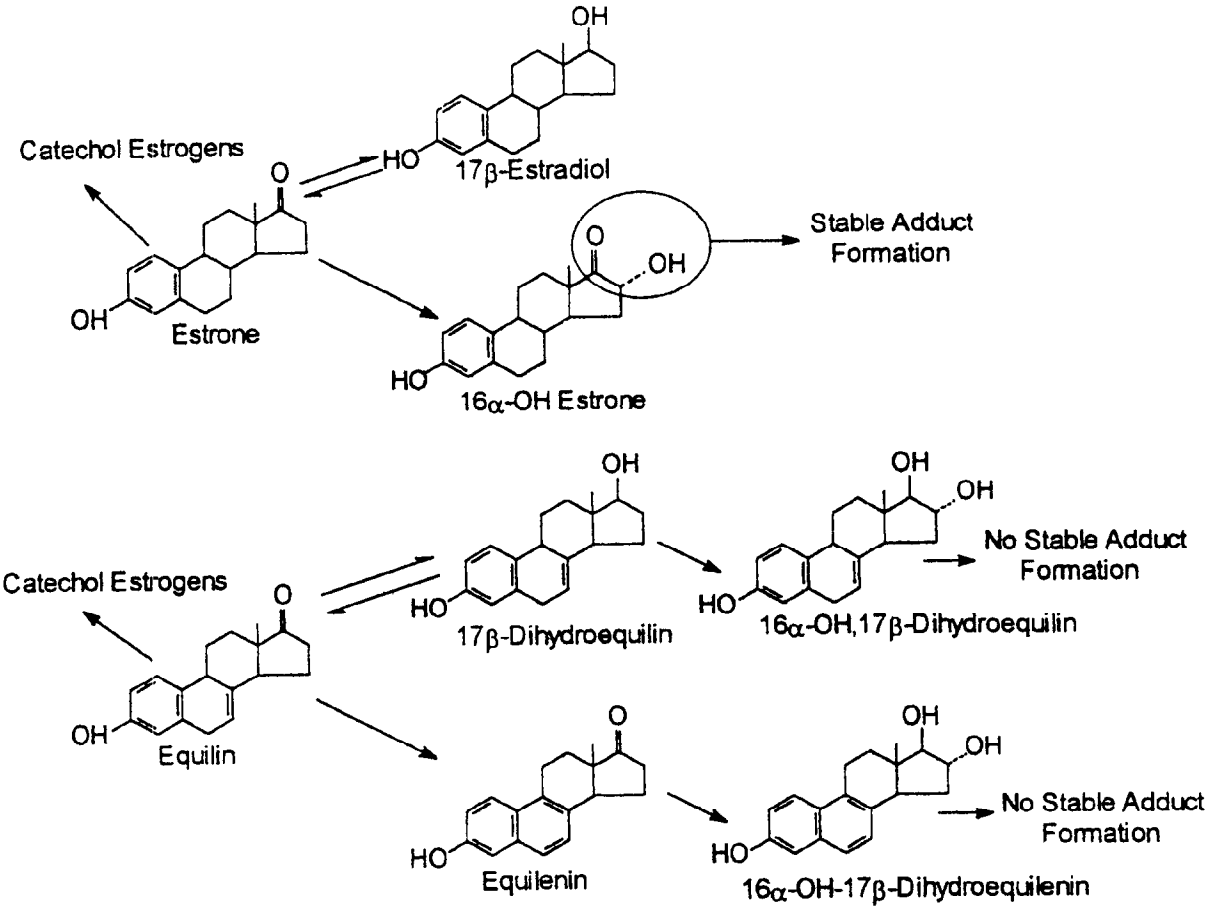

Figure 7. *In vivo* 16α-hydroxylation of estrone and the ring B unsaturated estrogen equilin, and the potential for forming adducts.

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."

I would like to express my sincere thanks to Drs. Allan Woolever, Anthony Cecutti, and Allan Gerulath for their continuing enthusiasm and support. The word processing skills of Mrs. Francine Bhavnani and Jean-Pierre Bhavnani in the preparation of this manuscript are highly appreciated.

1. Bhavnani BR, Woolever CA. *In vivo* metabolism of [^{3}H]equilin in the pregnant mare. Endocrinology **108**:232–238, 1981.
2. Bhavnani BR. The saga of the ring B unsaturated equine estrogens. Endocrine Rev **9**:396–416, 1988.
3. Dorfman RI, Dorfman AS. The assay of estrogens in the chick by oral administration. Endocrinology **53**:301–305, 1953.
4. Dorfman RI, Dorfman AS. Estrogen assays using the rat uterus. Endocrinology **55**:65–69, 1954.
5. Howard RP, Keaty KC. Evaluation of equilin-3-monosulfate and other estrogens. Arch Int Med **128**:229–233, 1971.
6. Beek VA, Friedrich F. Equilin sulfate zur substitution beim menopause-syndrom. Wien Klin Wochenschr **87**:59–62, 1975.
7. Lobo RL, Nguyen HN, Eggena P, Brenner PF. Biologic effects of equilin sulfate in postmenopausal women. Fertil Steril **49**:234–238, 1988.
8. Artner J, Friedrich F. Die behandlung des klimakerichen syndroms mit dihydroequilin. Wien Klin Wochenschr **82**:275–277, 1970.
9. Bhavnani BR, Cecutti A, Dey MS. Effect in postmenopausal women of delta-8-estrone sulfate: a novel estrogen component of premarin. J Soc Gynecol Investig **4**:(No. 1, Supplement) 177A (Abstract No. 392) 1997.
10. Mashchak CA, Lobo RA, Dozono R, Eggena P, Nakamura RM, Brenner PF, Mikhail G. Comparison of pharmacodynamic properties of various estrogens formulations. Am J Obstet Gynecol **144**:511–518, 1982.
11. Evans RM. The steroid and thyroid hormone receptor superfamily. Science **240**:889–895, 1988.
12. Bhavnani BR, Woolever CA. Interaction of ring B unsaturated estrogens with estrogen receptors of human endometrium and rat uterus. Steroids **56**:201–209, 1991.
13. Bhavnani BR, Gerulath A, Cecutti A. Pharmacokinetics and pharmacodynamics of delta-8-estrone. J Soc Gynecol Investig **3**(Suppl. 2) (Abstract No. 613), 1996.
14. Yang NN, Venugopalan M, Hardikor S, Glasebrook A. Identification of an estrogen response element activated by metabolites of 17β-estradiol and raloxifene. Science **273**:1222–1225, 1996.
15. Pan CC, Woolever CA, Bhavnani BR. Transport of equine estrogens: Binding of conjugated and unconjugated equine estrogens with human serum proteins. J Clin Endocrinol Metab **61**:499–507, 1985.
16. Rosenthal H, Pietrzak E, Slaunwhite WR Jr., Sandberg AA. Binding of estrone sulfate in human plasma. J Clin Endocrinol Metab **34**:805–813, 1972.
17. Wu CH, Motohashi T, Abdel-Rahman HA, Flickinger GL, Mikhail G. Free and protein bound plasma estradiol 17β during the menstrual cycle. J Clin Endocrinol Metab **43**:436–445, 1976.
18. Dunn JF, Nisula BC, Rodbard D. Transport of steroid hormones: Binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid binding globulin in human plasma. J Clin Endocrinol Metab **53**:58–68, 1981.
19. Bhavnani BR, Sarda IR, Woolever CA. Radioimmunoassay of plasma equilin and estrone in postmenopausal women after the administration of Premarin. J Clin Endocrinol Metab **52**:741–747, 1981.
20. Ruder HJ, Loriaux DL, Lipsett MB. Estrone sulfate: Production rate and metabolism in man. J Clin Invest **51**:1020–1033, 1972.
21. Anderson ABM, Sklovsky E, Sayers L, Steele P, Turnbull AC. Comparison of serum oestrogen concentrations in postmenopausal women taking estrone sulphate and oestradiol. BMJ **1**:140–142, 1978.
22. Bhavnani BR, Woolever CA, Wallace D, Pan CC. Metabolism of [^{3}H]equilin-[^{35}S]sulfate and [^{3}H]equilin sulfate after oral and intravenous administration in normal postmenopausal women and men. J Clin Endocrinol Metab **68**:757–765, 1989.
23. Longcope C. The metabolism of estrone sulfate in normal males. J Clin Endocrinol Metab **34**:113–122, 1972.
24. Tait JF, Burstein S. *In vivo* studies of steroid dynamics in man. In: Pincus G, Thimann KV, Astwood EB, Eds. The Hormones. New York: Academic Press, pp 441–557, 1964.
25. Bhavnani BR, Woolever CA, Benoit H, Wong T. Pharmacokinetics of equilin and equilin sulfate in normal postmenopausal women and men. J Clin Endocrinol Metab **56**:1048–1056, 1983.

26 Bhavnani BR, Cecutti A. Pharmacokinetics of 17β-dihydroequilin sulfate and 17β-dihydroequilin in normal postmenopausal women. J Clin Endocrinol Metab 78:197–204, 1994

27 Bhavnani BR, Cecutti A. Metabolic clearance rate of equilin sulfate and its conversion to plasma equilin, conjugated and unconjugated equilenin, 17β-dihydroequilin, and 17β-dihydroequilenin in normal postmenopausal women and men under steady state conditions. J Clin Endocrinol Metab 77:1269–1274, 1993.

28 Bhavnani BR, Cecutti A, Gerulath AH. 17β-Dihydroequilin sulfate dynamics in postmenopausal women and men under steady state conditions. J Soc Gynecol Investig 4:No 1 (Suppl.) 177A (Abstract 391), 1997.

29 Sandberg E, Gurpide E, Lieberman S. Quantitative studies on the metabolism of dehydroisoandrosterone sulfate. Biochemistry 3:1256–1267, 1966.

30. Wang DY, Bulbrook RD, Sheedon A, Hamilton T. The metabolic clearance rates of dehydroepiandrosterone, testosterone and their sulphate esters in man, rat and rabbit. J Endocrinol 38:307–318, 1967.

31. Longcope C, Tait JF. Validity of metabolic clearance rates and interconversion rates and transfer factors. J Clin Endocrinol Metab 32:481–490, 1971.

32. Longcope C. Metabolic clearance and blood production rates of estrogens in postmenopausal women. Amer J Obstet Gynecol 111:778–781, 1971.

33. Longcope C, Layne DS, Tait JF. Metabolic clearance rates and interconversions of estrone and 17β-estradiol in normal males. J Clin Invest 47:93–106, 1968.

34. Hembree WC, Bardin CW, Lipset MB. A study of estrogen metabolic clearance rates and transfer factors. J Clin Invest 48:1809–1813, 1969.

35. Bhavnani BR, Cecutti A, Wallace D. Metabolism of [^{3}H]17β-dihydroequilin and [^{3}H]dihydroequilin-dihydroequilin sulfate in normal postmenopausal women. Steroids 59:389–394, 1994.

36. Bhavnani BR, Woolever CA. The metabolism of equilin in normal men. J Steroid Biochem Mol Biol 17:217–223, 1982.

37. Bhavnani BR, Gerulath AH. Metabolism of [^{3}H]equilin in normal and malignant human endometrium and in endometrial adenocarcinoma transplanted into nude mice. J Steroid Biochem Mol Biol 38:433–439, 1991.

38. Ryan KJ, Engle LL. The interconversion of estrone and estradiol in human tissue slices. Endocrinology 52:287–291, 1953

39. Tseng L, Gurpide E. Estradiol and 20α-dihydroprogesterone dehydrogenase activities in human endometrium during the menstrual cycle. Endocrinology 94:419–423, 1974.

40 Purdy RH. Active intermediates and carcinogenesis. In: Merriam YR, Ed. Catechol Estrogens. New York: Raven Press, pp 123–140, 1983

41 Zhu BT, Roy D, Liehr JG. The carcinogenic activity of ethinyl estrogens is determined by both their hormonal characteristics and their conversion to catechol metabolites. Endocrinology 132:577–583, 1993.

42. Li JJ, Li SA. Estrogen carcinogenesis in hamster tissues. Endocrine Rev 11:524–531, 1990

43 Li JJ, Li SA, Oberley TD, Parson JA. Carcinogenic activities of various steroidal and non-steroidal estrogens in the hamster kidney. Relation to hormonal activity and cell proliferation. Cancer Res 55:4347–4351, 1995.

44. Bhavnani BR, Lau A, Cecutti A, Gerulath A. Demonstration of 2- and 4-hydroxylation of equilin by normal proliferative and secretory human endometrial microsomal preparations. J Soc Gynecol Investig 2:424 (Abstract No 415), 1995.

45. Reddy VVR, Hanjani P. Synthesis of catechol estrogens by human uterus and leiomyoma. Steroids 37:195–199, 1981

46. Bhavnani BR, Cecutti A. Identification of novel 16α-hydroxylated ring B unsaturated estrogens following administration of equilin to postmenopausal women and men. J Soc Gynecol Investig 3(Suppl 2):365 (Abstract No. 615), 1996.

47. Schneider J, Kinne D, Fracchia A, Pierce V, Anderson KE, Bradlow HL, Fishman J. Abnormal oxidative metabolism of estradiol in women with breast cancer. Proc Natl Acad Sci (USA) 79:3047–3051, 1982.

48. Bradlow HL, Hershcopf RJ, Martucci CP, Fishman J. Estradiol 16α-hydroxylation in the mouse correlates with mammary tumor incidence and presence of marine mammary tumor virus: A possible model for the hormonal etiology of breast cancer in humans. Proc Natl Acad Sci (USA) 82:6295–6299, 1985.

49. Swaneck GE, Fishman J. Covalent binding of the endogenous estrogen 16α-hydroxy estrone to estradiol receptor in human breast cancer cells: Characterization and intranuclear localization. Proc Natl Acad Sci (USA) 85:7831–7835, 1988

50. Yu SC, Fishman J. Interaction of histones with estrogens: Covalent adduct formation with 16α-hydroxy-estrone. Biochemistry 24:8017–8021, 1985.

51. Miyairi S, Ichikawa T, Nambara T. Structure of the adduct of 16α-hydroxy estrone with a primary amine: Evidence for the Heyns rearrangement of steroidal D-ring-hydroxymines. Steroids 56:361–366, 1991.

Biologic Effects of Delta-8-Estrone Sulfate in Postmenopausal Women

Bhagu R. Bhavnani, PhD, Anthony Cecutti, MD, and Mike S. Dey, PhD

OBJECTIVE: *To determine in postmenopausal women the biological effects of delta-8-estrone sulfate, a novel estrogen component of Premarin (Wyeth-Ayerst, Philadelphia, PA).*

METHODS: *An open-label, nonrandomized study of six healthy postmenopausal women was conducted. Each subject took 0.125 mg of delta-8-estrone sulfate daily for 8 weeks. Blood samples were collected at day 0 (baseline) and once a week for 8 weeks. Urine was collected on day 0 and at weeks 2, 3, 5, 6, 7, and 8. Serum gonadotropins (follicle-stimulating hormone/luteinizing hormone), plasma binding proteins (corticosteroid-binding globulin/sex hormone–binding globulin), a marker for bone turnover (urinary n-telopeptide), and markers for cardiovascular effects (cholesterol, low-density lipoprotein$_c$, high-density lipoprotein$_c$, low-density lipoprotein oxidation, and rate of diene formation) were measured.*

RESULTS: *Follicle-stimulating hormone levels decreased from 84.0 ± 8.5 to 67.0 ± 8.5 mIU/mL (P = .02), whereas luteinizing hormone levels did not change. Corticosteroid-binding globulin levels increased from 3.30 ± 0.16 to 4.10 ± 0.16 mg/dL (P = .02), and no change in sex hormone–binding globulin was noted. The n-telopeptide levels decreased an average of 31% from 40.7 ± 4.9 to 28 ± 7.0 nmol/L bone collagen equivalents/mmol/L creatinine (P = .03). Plasma diene concentration and diene production rate decreased by 34% and 40%, respectively; these changes were not significantly different from baseline values. In contrast, a significant (P = .03) 68% increase in the lag time for low-density lipoprotein$_c$ oxidation (38.5 ± 5.5 minutes versus 64.8 ± 8.5 minutes) was observed. No significant change occurred in total cholesterol, high-density lipoprotein$_c$, and low-density lipoprotein$_c$.*

CONCLUSION: *Small doses (0.125 mg) of delta-8-estrone sulfate have profound estrogenic effects in postmenopausal women. The changes observed in n-telopeptide levels and the lag-time delay in oxidation of low-density lipoprotein$_c$ indicate that this estrogen contributes toward the overall beneficial effects on bone and cardiovascular system associated with Premarin therapy. (J Soc Gynecol Invest 1998;5:156–160)*

KEY WORDS: Delta-8-estrone sulfate, oxidized low-density lipoprotein, oxidation lag time, n-telopeptide.

Premarin (Wyeth-Ayerst, Philadelphia, PA), a conjugated equine estrogen preparation, has been extensively used for estrogen replacement therapy, prevention of osteoporosis, and cardiovascular disease in postmenopausal women. Premarin has been known to contain[1] the sulfate esters of the ring B saturated 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), and 17β-dihydroequilenin (17β-Eqn: 1,3,5(10)6,8-estrapentaen-3,17β diol). Along with the above nine estrogens, the presence of a tenth ring B unsaturated estrogen component identified as delta-8-estrone (delta-8-E_1, Isoequilin; 3-hydroxy-1,3,5(10), 8-estratetraen-17-one) has now been confirmed in Premarin. Delta-8-E_1 is the fifth most abundant estrogen component in Premarin and makes up approximately 3.5% of the total estrogen present in a Premarin tablet. Recently,[2] it was demonstrated that delta-8-E_1 has in vivo estrogenic activity, can interact with uterine estrogen receptors, and that after intravenous administration in men and postmenopausal women, it was cleared from the blood fairly rapidly (metabolic clearance rate: 1711 ± 252 L/d/m^2). The in vivo pattern of metabolism of delta-8-E_1 appears to be quite unique in that the major circulating metabolite in both the unconjugated and the sulfate conjugated form was the 17β-reduced estrogen: delta-8, 17β-estradiol (delta-8, 17β-E_2: 1,3,5,(10),8-estratetraen-3, 17β-diol). In this study, we have determined the in vivo effects of daily oral administration of 0.125 mg of delta-8-estrone sulfate (delta-8-E_1S), in post-

From the Department of Obstetrics and Gynecology and Institute of Medical Sciences and St. Michael's Hospital, University of Toronto, Toronto, Ontario; and Wyeth-Ayerst Research, Philadelphia, Pennsylvania

This work was supported by the Medical Research Council of Canada (grants MT3724 and MT11329) and Wyeth-Ayerst Research.

Address all correspondence to and reprint requests to: Bhagu R Bhavnani, PhD, Department of Obstetrics and Gynecology, St Michael's Hospital, 30 Bond Street, Toronto, Ontario, M5B 1W8, Canada.

Published by Elsevier Science Inc.

1071-5576/98/$19.00
PII S1071-5576(98)00003-3

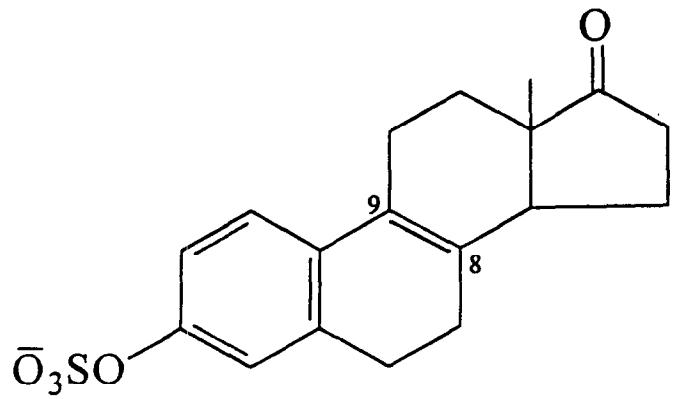

Figure 1. Structure of delta-8-E_1S.

menopausal women. This is the amount of delta-8-E_1S present in one 2.5 mg Premarin tablet.

MATERIALS AND METHODS

Study Design

This trial was an open-label, nonrandomized pilot study designed to determine the effects of daily oral administration of 0.125 mg delta-8-E_1S (Figure 1) for 8 weeks, on serum gonadotropins (follicle-stimulating hormone [FSH]/luteinizing hormone [LH]); plasma binding proteins (corticosteroid-binding globulin [CBG], sex hormone–binding globulin [SHBG]), a marker for bone turnover (urinary N-telopeptide), and markers for cardiovascular effects such as cholesterol, low-density lipoprotein$_c$ (LDL_c), high-density lipoprotein$_c$ (HDL_c), LDL oxidation rate, and diene formation in postmenopausal women.

Subjects

Six healthy postmenopausal women, between the ages of 47 and 60 years, were recruited to participate in the study. All subjects were nonsmokers and had undergone natural menopause and postmenopausal amenorrhea for at least 12 months. They were not taking any hormones or antioxidant supplements or experiencing vasomotor symptoms and their body weight was within ±20% of ideal weight. Their 17β-E_2 and FSH were less than 20 pg/mL and greater than 50 mIU/mL, respectively. This study was approved by the Human Research Ethics Board of St. Michael's hospital and the University of Toronto. Informed consent was signed by each subject before initiation of the study.

Sample Collection and Analysis

Blood samples were collected after overnight fasting at day zero (predose, baseline) and, following administration, once a week for 8 weeks. Plasma (ethylenediamine tetra-acetic acid 2 mg/mL) was refrigerated at 4C and shipped within 24 hours for analysis of HDL_c, LDL_c, total cholesterol, conjugated dienes, diene production rate, and LDL oxidation (lag-time determination). Serum was stored at −20C until shipped (dry ice) for analysis of CBG, SHBG, FSH, and LH. First morning void urine samples were collected in amber glass vials on day 0 and at weeks 2, 4, 6, and 8. These urine samples were stored at −20C until shipped (dry ice) for analysis of n-telopeptide. All plasma, serum, and urine samples were analyzed in a blinded manner by university/hospital-based clinical research laboratories or commercial laboratories as follows: serum FSH, LH (immunochemilluminometric assays), binding capacity of SHBG (displacement technique), serum CBG (radioimmunoassay), and 17β-E_2 (radioimmunoassay after extraction and LH_{20} column chromatography) were assayed by Endocrine Sciences, Agoura Hills, CA. The intra- and interassay coefficient of variation for all of the above assays were less than 10%. The following assays were performed in Dr. Jean Davignons' research laboratories, Clinical Research Institute of Montreal, University of Montreal, Montreal, Canada. Plasma cholesterol and HDL (isolated by the heparin-magnanese separation of apo B-containing lipoproteins) were measured enzymatically[3] on an automated autoanalyzer. The value for LDL cholesterol was calculated using the equation of Friedewald et al.[4] Plasma levels of conjugated dienes were determined by a spectrophotometric assay.[5] The LDL oxidation (lag time) and the propagation rate of conjugated diene production were measured by continuous monitoring at 234 nm as described by Esterbauer et al.[6] Type 1 collagen cross-linked n-telopeptide levels in urine were measured using an enzyme-linked immunosorbent assay (ELISA) immunoassay kit[7] at the Bone Metabolism Department, Helen Hayes Hospital, NY. This assay has been used to monitor and predict therapeutic effects of estrogen replacement therapy on bone mineral density in postmenopausal women.[8] The n-telopeptide values have been normalized on creatinine clearance.

Statistical Analysis

Baseline values were compared with values obtained after the last week of therapy by using paired Student *t* tests. Paired data from each subject were used for these statistical analyses, however, in the figures, the mean ± standard error of baseline and final week treatment values from all six subjects are depicted. $P \leq .05$ was considered significant.

RESULTS

All subjects tolerated the dose of 0.125 mg of delta-8-E_1S taken daily for 8 weeks. None of the subjects experienced vaginal bleeding, spotting, withdrawal bleeding, or any other side effects. One subject was out of town during week 8 and; therefore, she continued to take the delta-8-E_1S for an additional week.

Follicle-Stimulating Hormone/Luteinizing Hormone

Serum FSH decreased significantly ($P < .02$) from a mean 84.0 ± 8.5 mIU/mL (range: 64–108) to a mean value of 67.0 ± 8.5 mIU/mL (range: 44–104) after 8 weeks of therapy with delta-8-E_1S. In contrast, no significant change in LH levels was observed between the baseline 42.8 ± 8.5 mIU/mL (range: 27–81) and post-treatment levels 40.5 ± 7.5 mIU/mL (range: 26–64).

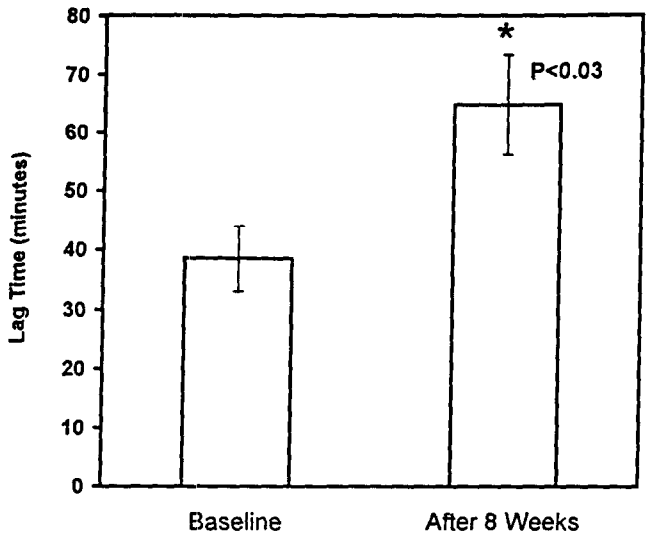

Figure 2. Changes in the lag time (LDL oxidation) after daily oral administration of 0.125 mg of delta-8-E_1S to postmenopausal women

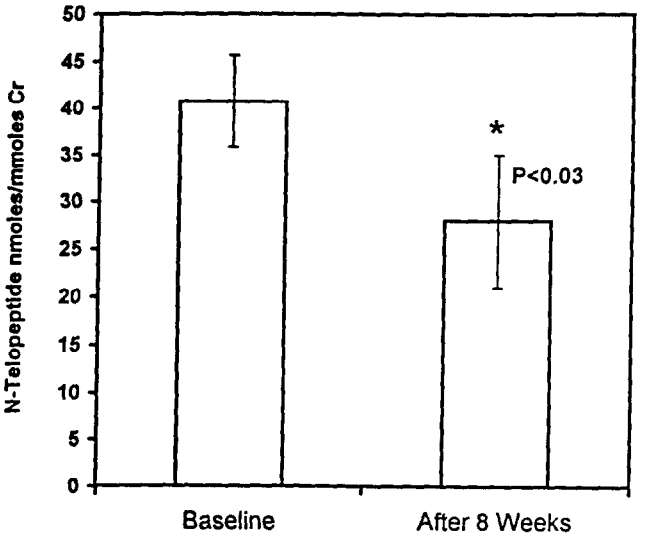

Figure 3. Changes in urinary n-telopeptide concentrations after daily oral administration of 0.125 mg of delta-8-E_1S to postmenopausal women.

Corticosteroid-Binding Globulin/Sex Hormone–Binding Globulin

Though serum CBG levels significantly increased ($P < .02$), from a mean baseline value of 3.3 ± 0.16 mg/dL (range: 2.7–3.9) to 4.10 ± 0.16 mg/dL (range: 3.4–4.6) after 8 weeks of therapy, no significant change was observed in the serum SHBG levels (baseline: 1.2 ± 0.18 μg/dL versus post treatment value 1.15 ± 0.15 μg/dL).

Markers of Cardiovascular Effects (Lipid Parameters)

During the 8-week treatment with delta-8-E_1S, total plasma cholesterol levels did not significantly change from baseline levels 5.255 ± 0.200 mmol/L (range: 4.55–6.01) versus the post-treatment value (5.15 ± 0.37 mmol/L) (range: 3.7–6.3). Though a 12% increase in HDL cholesterol (1.30 ± 0.16 mmol/L versus 1.5 ± 0.2 mmol/L) and a 6% decrease in LDL cholesterol (3.0 ± 0.2 mmol/L versus 2.8 ± 0.4 mmol/L) were observed after the 8-week treatment period, these changes were not significantly different from the baseline values. Plasma diene concentrations decreased by 34% from the baseline levels of 0.25 ± 0.03 mmol/L to 0.165 ± 0.020 mmol/L, and similarly, the diene production rate decreased by 40% from 11.2 ± 2.2 nmol/min/mg protein to 6.7 ± 0.6 mmol/min/mg protein, these changes were not statistically significant with $P > 0.1$ and $P > 0.09$, respectively. In contrast, as depicted in Figure 2, a significant 68% ($P < .03$) increase in the lag time for the LDL oxidation from a baseline value of 38.5 ± 5.5 minutes versus 64.8 ± 8.5 minutes post-treatment, was observed.

Urinary N-Telopeptides

The levels of urinary n-telopeptides of type 1 collagen (Figure 3) expressed as n moles bone collagen equivalents per mmole creatinine (nM BCE/nM Cr), decreased by 31% ($P < .03$) from the baseline values of 40.7 ± 5.0 to 28 ± 7 nM BCE/nM Cr after 8 weeks of therapy with delta-8-E_1S.

DISCUSSION

In this study, we provide data that indicate that the novel ring B unsaturated estrogen delta-8-E_1S is a potent estrogen. A dose of 0.125 mg of delta-8-E_1S administered daily for 8 weeks significantly increased CBG, and the lag time for LDL oxidation and decreased FSH and the urinary levels of the bone resorption marker n-telopeptide. The lack of significant changes in other estrogen-induced end points such as SHBG, cholesterol, LDL_c, and HDL_c are most likely due to the short duration of this study and/or the small number of subjects. Although the dose (0.125 mg) of delta-8-E_1S used in this study is the amount present in one 2.5-mg tablet of Premarin, this is still a relatively very small amount of oral estrogen to have biological effects in postmenopausal women. Other individual ring B unsaturated estrogen components of Premarin have also been shown to have biologic effects. Thus, Howard and Keaty[9] demonstrated that in postmenopausal women, oral EqS was five times more potent in suppression of urinary gonadotropins than E_1S. Similarly, 0.25 mg of EqS was as active as 0.625 mg of Premarin in stimulating the vaginal epithelium and alleviating menopausal vasomotor symptoms.[10] Lobo et al,[11] also demonstrated that doses of 0.31 and 0.625 mg of EqS were 1.5 to 8.0 times more potent than comparable doses of E_1S and conjugated equine estrogens (Premarin) in stimulating the synthesis of hepatic proteins such as SHBG, CBG, angiotensinogen, and HDL_c. However, EqS alone was somewhat less potent than Premarin in its ability to inhibit bone resorption, but its activity was similar to that of E_1S. In this study, 0.125 mg of delta-8-E_1S was found to significantly lower the levels of the urinary bone resorption marker n-telopeptide. These findings suggest that on direct weight basis (ie, milligram

for milligram) this estrogen may be a more potent inhibitor of bone resorption. However, more detailed dose-response studies are needed before firm conclusions can be made. An earlier study[12] showed that the 17β-reduced metabolites of ring B unsaturated estrogens such as 17β-EqS were also effective in controlling vasomotor symptoms. In this study, oral administration of 0.3–0.4 mg/d of 17β-EqS was as effective as 0.625 mg of conjugated equine estrogens. In a previous study,[2] it was demonstrated that the pattern of in vivo metabolism of delta-8-E_1 was rather unique, in that the major metabolite in both the unconjugated and the sulfate conjugated form was the more active 17β-reduced product. Though the extent of 17β-reduction, ie, formation of delta-8–17β-E_2 and delta-8–17β-E_2S, is in keeping with observations with other ring B unsaturated estrogens, the extent of this activation was much greater.[13] Because it has been previously reported[2] that delta-8-E_1 in postmenopausal women was metabolized mainly to delta-8–17β-E_2S and delta-8–17β-E_2, it is quite possible that some of the biologic effects observed in this study may be due to the formation of these active metabolites. Similarly, it has also been suggested that the biologic effects of EqS may be due to the formation of its active metabolites 17β-Eq and 17β-EqS.[1,14]

Though the cardioprotective effects of estrogen replacement[15–17] and hormone replacement (estrogen + progestin) therapy[18] in postmenopausal women have been well documented, the mechanism for this beneficial effect has not been established. An earlier hypothesis proposed that estrogen use was associated with hepatic lipid changes that result in decreased levels of total cholesterol and LDL_c and an increase in HDL_c and that these were the important factors. However, more recent studies indicate that these lipid changes account for only 25–50% of the reduction in cardiovascular risk associated with estrogen therapy.[16] More recent studies indicate that oxidatively modified LDL plays an important role in the process of atherogenesis and that oxidized LDL is more atherogenic.[19] Inhibition of the formation of oxidized LDL would therefore be beneficial. A number of in vitro and in vivo studies[20–26] have shown that various estrogens can inhibit oxidation of LDL. An in vitro study[26] also indicated that delta-8-estrone, 17α-dihydroequilenin, and 17β-dihydroequilenin were the most potent antioxidants present in Premarin. Data from the present in vivo study clearly indicate that small amounts of delta-8-E_1S administered orally for only 8 weeks also inhibited the oxidation of LDL (increase in lag time, Figure 2). Thus, both in vitro and in vivo observations indicate that delta-8-E_1 is a potent antioxidant.

Administration of Premarin to postmenopausal women[27,28] also resulted in a significant inhibition of LDL oxidation, and because all ten known components of Premarin have antioxidant properties,[25] some of the cardiovascular benefits associated with this drug are most likely due to this mechanism. Furthermore, the results from this study, with regard to n-telopeptide levels and the lag-time delay in the oxidation of LDL indicate that delta-8-E_1S contributes significantly to the overall beneficial effects on bone and cardiovascular system associated with Premarin therapy. These data further support the hypothesis that the biological effects of Premarin are the sum of the effects of its various individual components.[1]

REFERENCES

1. Bhavnani BR. The saga of the ring B unsaturated equine estrogens. Endocr Rev 1988;9:396–416.
2. Bhavnani BR, Gerulath A, Cecutti A. Pharmacokinetics and pharmacodynamics of delta-8-estrone. J Soc Gynecol Invest 1996;3:(Suppl 2):364A.
3. Allain CB, Poon LS, Chan FCS, Fu PC. Enzymatic determination of total serum cholesterol. Clin Chem 1974;20:470–5.
4. Friedewald WT, Levy RI, Fredickson DS. Estimation of the concentration of low density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972; 18:499–502.
5. Quintanilha AT, Packer L, Szyszlo Davies JM, Racanelli TL, Davies KJA. Membrane effects of vitamin E deficiency Bioenergetic and surface charge density studies of skeletal muscle and liver mitochondria. Ann N Y Acad Sci 1986;393:32–47.
6. Esterbauer H, Striegl G, Puhl H, Rotheneder M Continuous monitoring of *in vitro* oxidation of human low density lipoprotein. Free Radic Res 1989;6:67–75.
7. Hanson DA, Weis MAE, Bollen AM, Maslan SL, Singer FR, Eyre DR. A specific immunoassay for monitoring human bone resorption: Quantitation of type 1 collagen cross-linked n-telopeptide in urine. J Bone Miner Res 1992;7:1251–8.
8. Chesnut CH, Bell NH, Clark GS, et al. Hormone replacement therapy in postmenopausal women: Urinary n-telopeptide of type 1 collagen monitors therapeutic effect and predicts response of bone mineral density. Am J Med 1997;102:29–37
9. Howard RP, Keaty KC. Evaluation of equilin-3-monosulfate and other estrogens. Arch Int Med 1971;128:229–34.
10. Beek VA, Friedrich F. Equilin sulfat zur substitution beim menopause-syndrom. Wien Klin Wochensch 1975,87·59–62
11. Lobo RL, Nguyen HN, Eggena P, Brenner PF Biologic effects of equilin sulfate in postmenopausal women. Fertil Steril 1988; 49:234–338.
12. Artner J, Friedrich F. Die behandlung des klimaklerichen syndroms mit dihydroequilin. Wien Klin Wochensch 1970;82:275–7.
13. Bhavnani BR. Pharmacokinetics and pharmacodynamics of conjugated equine estrogens: Chemistry and metabolism. Proc Soc Exp Biol Med 1998;217:6–16.
14. Bhavnani BR, Cecutti A. Metabolic clearance rate of equilin sulfate and its conversion to plasma equilin, conjugated and unconjugated equilenin, 17β-dihydroequilin, and 17β-dihydroequilenin in normal postmenopausal women and men under steady state conditions. J Clin Endocrinol Metab 1993;77:1269–74.
15. Stampfer MJ, Colditz GA, Willett WC, et al. Postmenopausal estrogen therapy and cardiovascular disease. N Engl J Med 1991; 325:756–62.
16. Bush TL, Barrett-Conner E, Cowan LD, et al. Cardiovascular mortality and noncontraceptive use of estrogens in women Results from the Lipid Research Clinics Programs Follow-up Study. Circulation 1987;75:1102–9.
17. Sullivan JM, Fowlkes LP. The clinical aspects of estrogen and cardiovascular system. Obstet Gynecol 1996;87(Suppl 2) 36S–43S.
18. Grodstein F, Stampfer M, Manson JE, et al. Postmenopausal estrogen and progestin use and the risk of cardiovascular disease. N Engl J Med 1996;335:453–61.
19. Steinberg F, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol: Modifications of low-density lipoprotein that increases its atherogenicity. N Engl J Med 1989;320:915–24.

20. Huber LA, Scheffler E, Poll T, Ziegler R, Dressel HA. 17β-estradiol inhibits LDL oxidation and cholesterol ester formation in cultured macrophages. Free Radic Res 1990;8:167–75.
21. Maziere C, Auclair M, Ronveaux MC, Salmon S, Santus R, Maziere JC. Estrogens inhibit copper and cell-mediated modifications of low density lipoprotein. Atherosclerosis 1991;89:175–82.
22. Subbiah MTR, Kessel B, Agrawal M, et al. Antioxidant potential of specific estrogens on lipid peroxidation. J Clin Endocrinol Metab 1993;77:1095–7.
23. Sack MN, Rader DJ, Cannon RO. Oestrogen and inhibition of low density lipoproteins in postmenopausal women. Lancet 1994;343:269–70.
24. Tang M, Abplenalp W, Ayres S, Subbiah MTR. Superior and distinct antioxidant effects of selected estrogen metabolites on lipid peroxidation. Metabolism 1996;45:411–4.
25. Wilcox JG, Sevanian A, Hwang J, Stanczyk FZ, Hodis HN, Lobo RA. Cardioprotective effects of individual conjugated equine estrogens through their possible modulation of insulin resistance and oxidation of low-density lipoprotein. Fertil Steril 1997;67:57–62.
26. Bhavnani BR, Cecutti A, Gerulath A, Woolever CA. Are cardiovascular benefits associated with conjugated equine estrogen therapy and red wine consumption a result of the antioxidant properties of their components? J Soc Gynecol Invest 1997;4 (Suppl 1):106A.
27. Wilcox JG, Hwang J, Gentzschein EK, et al. Effects of combined estrogen and progestin therapy in postmenopausal women on endothelin levels and oxidation of LDL. J Soc Gynecol Invest 1996;3(Suppl 2):69A.
28. Bhavnani BR, Cecutti A. Comparison of antioxidant effects of Premarin, Premarin plus progestin and generic conjugated estrogen (C.E.S.) administration in postmenopausal women. J Soc Gynecol Invest 1997;4(Suppl 1):176A.

0021-972X/83/5605-1048$02 00/0
Journal of Clinical Endocrinology and Metabolism

Vol 56, No. 5
Printed in U S A

Pharmacokinetics of Equilin and Equilin Sulfate in Normal Postmenopausal Women and Men*

BHAGU R. BHAVNANI, C. ALLAN WOOLEVER, HENRY BENOIT, AND TONY WONG

Department of Obstetrics and Gynecology, McMaster University Health Sciences Centre, Hamilton, Ontario, Canada

ABSTRACT. The MCRs of equilin sulfate and equilin were determined in normal postmenopausal women and a normal man by single iv injections of either [^{3}H]equilin sulfate or [^{3}H] equilin. After the administration of [^{3}H]equilin sulfate, blood was drawn at various time intervals, and the plasma obtained was fractionated into the unconjugated, sulfate, and glucuronide fractions. The bulk of radioactivity was present in the sulfate fraction, and from this, [^{3}H]equilin sulfate, [^{3}H]17β-dihydroequilin sulfate, [^{3}H]equilenin sulfate, and [^{3}H]17β-dihydroequilenin sulfate were isolated and purified, and their concentrations were measured. The disappearance of radioactivity from plasma as equilin sulfate can be described as a function that is the sum of two exponentials. The initial fast component (half-life, 5.2 ± 1.2 min) represents distribution and transfer from a space, with a mean volume of 12.4 ± 1.6 liters. The mean value for the rate constant of total removal from the initial volume is 163 ± 19 U/day, of which 15.8 ± 2% is irreversible. The mean half-life of the slower component of equilin sulfate is 190 ± 23 min, and the mean MCR is 176 ± 44 liters/day·m^2.

Similarly, after the administration of [^{3}H]equilin to a normal postmenopausal woman and a man, the disappearance of radioactivity from plasma as equilin could be fitted by a single straight line, consistent with a one-compartment system. The half-life of equilin was approximately 19–27 min, and the MCR of equilin was calculated to be 1982 liters/day/m^2 in the normal man and 3300 liters/day/m^2 in the normal postmenopausal woman. The bulk of [^{3}H]equilin was very rapidly metabolized to mainly equilin sulfate. Small amounts of 17β-dihydroequilin sulfate and 17β-dihydroequilin were also isolated from the plasma. The *in vivo* formation of 17β-dihydroequilin and its sulfate may be of importance, as this estrogen is approximately 8 times more potent as a uterotropic agent than equilin sulfate (*J Clin Endocrinol Metab* **56**: 1048, 1983)

EQUILIN sulfate is one of the major (25%) components of conjugated equine estrogen preparations such as Premarin (Ayerst Laboratories, Montreal, Canada), which also contains sulfate esters of estrone (45%), 17α-dihydroequilin (15%), 17α-dihydroequilenin, 17α-estradiol, 17β-estradiol, 17β-dihydroequilin, and 17β-dihydroequilenin (1, 2). These drugs have been in use for over 35 yr for treatment of postmenopausal women, and recently, a strong association between endometrial cancer and estrogen replacement therapy has been demonstrated in a number of case-control studies (3–5). Though the biological fate of estrone sulfate has been well established in man (6–10), similar information regarding ring B unsaturated estrogen equilin sulfate is not available. It has, however, been well established that the ring B unsaturated estrogens are excreted in the urine exclusively by the pregnant mare and are formed by a squalene-cholesterol-independent biosynthetic pathway (11–16). We have reported that equilin in the pregnant mare is metabolized and excreted in the urine primarily as equilin sulfate and its relatively inactive metabolite, 17α-dihydroequilin sulfate (17). We have also reported that after iv or oral administration of Premarin to postmenopausal women, some of the equilin sulfate and estrone sulfate present in Premarin appears in the serum as unconjugated equilin and estrone (18). In the present investigation, the pharmacokinetics of equilin sulfate and equilin in normal postmenopausal women and in men were determined by the administration of [^{3}H] equilin sulfate or [^{3}H]equilin by the single injection technique (19–21). The MCRs of the above two steroids as well as the metabolites of equilin sulfate present in blood were determined.

Received October 25, 1982
Address all correspondence and requests for reprints to Dr. B. R. Bhavnani, Department of Obstetrics and Gynecology, McMaster University, 1200 Main Street West, Hamilton, Ontario, L8N 3Z5 Canada
* This work was supported by grants from the Ontario Cancer Research and Treatment Foundation (Grant 368) and the Medical Research Council of Canada (Grant MT-3724)

Materials and Methods

Subjects

Five normal postmenopausal and castrate women, aged 38–60 yr, were studied. Subjects were in good health and had received no medication for at least 48 h before the experiment. Before this, the five postmenopausal women were on or had

been on exogenous estrogen replacement therapy (Premarin or Stilbesterol). All subjects had given informed consent before the procedure, and the project was approved by the Human Ethics Committees of the university and the hospital. For comparison and for the development of the methods, the experiments were initially performed in one healthy normal man (aged 58 yr).

Counting

The radioactivity was measured in a Nuclear-Chicago (Des Plaines, IL) or Beckman (Palo Alto, CA) liquid scintillation spectrometer. The radioactive material in crude urine and plasma was measured by counting 1-ml aliquots in 14 ml Biofluor (New England Nuclear Corp., Boston, MA) Crystalline samples were dissolved in 2 ml methanol to which were added 13 ml toluene phosphor solution containing 0.4% of Omnifluor (New England Nuclear). All other samples were counted using 15 ml toluene phosphor solution. All counts were corrected to 100% efficiency by external standardization.

Purification of steroids

Steroids were purified by chromatography (no. 3 MM paper, Whatman, Inc., Clifton, NJ) in the following systems (compositions expressed as vol/vol): A) isopropanol-*n*-butanol-ammonium hydroxide-water (45:15:1:39); B) heptane-methanol-H_2O (10:9:1); C) isooctane-methanol-H_2O (10:9:1); and D) benzene-cyclohexane-methanol-water (1 2·3·3).

Detection of estrogens

Estrogens were detected on paper with Pauly's reagent (22), which was prepared just before use by mixing equal volumes of sulfanilic acid (0.9 g sulfanilic acid plus 9 ml concentrated HCl plus 90 ml H_2O) with 5% $NaNO_2$ solution; this mixture was allowed to react for a few minutes, and then an equal volume of 10% Na_2CO_3 solution was added to it. The paper chromatograms are dipped in this mixture. Equilin and 17β-dihydroequilin gave orange-coloured spots, while equilenin and 17β-dihydroequilenin gave red-coloured spots

Isotopes

[2,4-^{3}H]Equilin (SA, 41 Ci/mmol; New England Nuclear Corp.), through custom synthesis, was the same preparation as that used previously (18). It was diluted with unlabeled equilin to give a specific activity of 130 mCi/mmol. Its radiochemical purity was found to be over 95% by isotope dilution techniques. [2,4-^{3}H]Equilin-3-sulfate, ammonium salt, was prepared by adding 500 μCi [2,4-^{3}H]equilin (SA, 130 mCi/mmol) dissolved in 0.5 ml dry pyridine to an ice-cold mixture of chlorosulfonic acid (0 05 ml) and pyridine (0.5 ml) After 15 min at 4 C, the reaction mixture was warmed to 70 C and then cooled to room temperature. The pyridinium salt of equilin sulfate formed was converted to the ammonium salt by dissolving the reaction product in 5 ml 7 N ammonium hydroxide. After the addition of 100 ml distilled water, the [^{3}H]equilin sulfate and the unreacted [^{3}H]equilin were extracted with 20 g Amberlite XAD-2 resin (23). The resin was first washed with water (15 ml; three times) and then with methanolic ammonia (50 ml; three times; 200 ml methanol plus 10 ml NH_4OH). The methanol eluate containing the steroids was evaporated to dryness, and the residue was partitioned between ether and water. The ether extract containing unreacted [^{3}H]equilin was discarded, and the [^{3}H]equilin sulfate present in the aqueous phase was recovered by reextraction with XAD-2 resin. The residue obtained (960×10^6 dpm) was chromatographed on paper using system A [isopropanol-*n*-butanol-ammonium hydroxide-water (45:15:1:39)]. A single peak of radioactive material (Rf = 0.25) corresponding in mobility to authentic equilin sulfate was present and was eluted with methanol and stored under nitrogen at −20 C. The radiochemical homogeneity of this material was established by the following criteria

Solvolysis and chromatography A small aliquot (1.5×10^6 dpm) of [^{3}H]equilin sulfate was solvolyzed in tetrahydrofuran and perchloric acid (24). A total of 1.45×10^6 dpm ether-soluble product (unconjugated equilin) was recovered (97%) Aliquots (1×10^5 dpm) of this material were chromatographed in systems B and C. A single peak of radioactive material corresponding in mobility to equilin was present in both systems.

Isotope dilution. Repeated crystallizations of an aliquot (2.85×10^5 dpm) of the solvolyzed material after dilution with carrier equilin (55 mg) indicated the radiochemical purity to be over 98% (X_1 = 5070; X_2 = 5170; X_3 = 5200; calculated specific activity, 5180 dpm/mg).

Pulse injection studies

These studies were carried out as described previously (19, 20). Subjects received, in an antecubital vein, between 30–60 μCi of either [2,4-^{3}H]equilin sulfate or [2,4-^{3}H]equilin in 10 ml 10% ethanol in isotonic saline solution. Aliquots of the injection solution were taken to determine the radioactivity administered. Heparinized blood samples (20 ml) were obtained at frequent intervals through an indwelling needle placed in a vein of the opposite arm. The samples were centrifuged, and the plasma was stored at −20 C until analyzed as described below.

Extraction of plasma

To monitor recoveries, known amounts (500–1000 μg) of carrier equilin, equilenin, 17β-dihydroequilin, and 17β-dihydroequilenin were added to each plasma sample. After equilibration for 30 min at room temperature, the plasma was extracted with ether (45 ml, three times). The ether phase was washed with water (15 ml; three times), and the ether was removed by flash evaporation. The ether extract containing the unconjugated steroids was stored at −20 C until further processing. Previous tests with [^{3}H]equilin had shown that over 99.5% of [^{3}H]equilin is removed by the ether, while under these conditions, less than 0.5% of steroid sulfates such as [^{3}H]equilin sulfate or [^{3}H]estrone sulfate was extractable. The ether-extracted plasma and the aqueous washes were combined, and any residual ether was removed under a stream of nitrogen. Known amounts of the above-mentioned four carrier estrogens were again added to the diluted plasma, the pH was adjusted

to 2, and the mixture was saturated with NaCl (20%) and extracted with freshly distilled tetrahydrofuran (150 ml; three times). The tetrahydrofuran extract containing the steroid conjugates was solvolyzed by the addition of 0.1% perchloric acid, followed by incubation at 37 C for 17 h. After neutralization with ammonium hydroxide, the tetrahydrofuran was evaporated under vacuum, and the aqueous phase remaining behind was extracted with ethyl acetate (100 ml; three times). The ethyl acetate phase was washed with water (30 ml; three times) and then evaporated to dryness. The residue containing steroids originally present as sulfates was fractionated into phenolic and neutral steroids by partition between benzene (25 ml) and 1 N NaOH (10 ml; four times). The benzene phase was washed with water (5 ml; six times), and the benzene was removed by evaporation under vacuum. This fraction, which contained the neutral steroids, was devoid of any radioactivity. The NaOH phase and aqueous washes were combined, their pH was adjusted to 6–7, and they were extracted with ether (100 ml; three times). The ether phase washed with water (30 ml; three times) and evaporated to dryness under vacuum. The residue contained over 98% of the radioactivity originally present. Insignificant counts remained in the aqueous phase, indicating the absence of glucuronides in the plasma. Previous tests using [^{3}H]equilin sulfate and [^{3}H]estrone sulfate indicated that the solvolysis under the above-described conditions was quantitative and, under these conditions, the [^{3}H]equilin formed was stable. Therefore, the addition of unconjugated carrier steroids instead of corresponding sulfate conjugates provided a reliable method for calculations of procedural losses.

Since preliminary experiments in which [^{3}H]equilin sulfate had been injected indicated that very small amounts of radioactivity in the plasma was in the unconjugated form, the first ether extract containing this fraction was not worked up any further.

Isolation and purification of labeled equilin and its metabolites from plasma

Each of the ether extract residues containing estrogens was chromatographed for 6 h on paper using system B. Four zones, corresponding in mobility to equilin (least polar), equilenin, 17β-dihydroequilin, and 17β-dihydroequilenin (most polar), were detected with Pauly's reagent, as described above. The zones corresponding in mobility to equilin and equilenin were rechromatographed for 65 h in system C. Single zones corresponding in mobility to equilin and equilenin were observed and eluted with a methanol-benzene (2.8) mixture. The eluates were filtered into glass-stoppered tubes and evaporated to dryness under nitrogen. Similarly, the 17β-dihydroequilin and 17β-dihydroequilenin zones were rechromatographed for 16 h in system D. Single zones corresponding in mobility to 17β-dihydroequilin and 17β-dihydroequilenin were observed and were eluted and processed as described above. The residue from these eluates was dissolved in methanol, and aliquots were assayed for radioactivity and mass as described below. The radiochemical purity of the chromatographically purified compounds was confirmed in selected instances by the isotope dilution technique, as shown in Table 1. These data indicated that the purification steps used were sufficient to attain radiochemical purity.

TABLE 1. Proof of radiochemical purity of ^{3}H-labeled estrogens isolated from plasma (sulfate fraction) after IV administration of [^{3}H] equilin-3-sulfate (subject 1)

Crystallization	SA (dpm/mg)			
	Equilin[a]	Equilenin[b]	17β-Dihydroequilin[c]	17β-Dihydroequilenin[d]
1	330	80	110	280
2	350	80	115	270
3	340	85	110	260
Calculated	330	80	110	270

[a] A total of 6600 dpm [^{3}H]equilin eluted from the paper chromatogram run in system C was mixed with 20 mg carrier equilin before crystallization. The calculated value is based on these figures.

[b] A total of 1600 dpm [^{3}H]equilenin eluted from the paper chromatogram run in system C was mixed with 20 mg carrier equilenin before crystallization.

[c] A total of 2220 dpm [^{3}H]17β-dihydroequilin eluted from the paper chromatogram run in system D was mixed with 20 mg carrier 17β-dihydroequilin before crystallization.

[d] A total of 5400 dpm [^{3}H]17β-dihydroequilenin eluted from the paper chromatogram run in system D was mixed with 20 mg carrier 17β-dihydroequilenin before crystallization.

Determination of procedural losses

The amount of carrier estrogens remaining after purification was determined by measuring the optical density at 280 nm using a Beckman model 25 spectrophotometer. The optical densities of the samples were compared with those obtained from known concentrations of equilin, equilenin, 17β-dihydroequilin, and 17β-dihydroequilenin. Knowing the mass of each carrier steroid added before extraction and the amount remaining after purification, the percent recovery was calculated (50–80%) and was used to correct the amount of radioactive estrogen isolated for procedural losses.

Analysis of data

The two-compartment model described by Tait *et al.* (19), Tait and Burstein (20), and Rizkallah *et al* (21) was used to calculate the MCRs of equilin and equilin sulfate. The MCR is defined as the volume of plasma irreversibly cleared of its hormone content per unit time (liters per day) and is also expressed in terms of surface area as liters per day/m^2.

The disappearance curve for radioactive equilin sulfate in plasma was represented by at least two exponentials $X' = A'e^{-\alpha t} + B'e^{-\beta t}$, where X' is the concentration of the radioactivity measured specifically as equilin sulfate, and

$$\text{MCR (liters/day)} = \frac{1}{\int_0^\infty X'dt} = \frac{1}{\int_0^\infty (A'e^{-\alpha t} + B'e^{-\beta t})\cdot dt}$$

$$= \frac{1}{\frac{A'}{\alpha} + \frac{B'}{\beta}} = \frac{\alpha\,\beta}{A'\beta + B'\alpha}$$

where A′ and B′ ar
by extrapolation o
the fraction of the
plasma), α is the
slope of the final p
volume into whicl
distributed, was ca
B′) at time zero
versible and irreve
lated using the equ
The percentage
calculated from t
moval/rate of tota

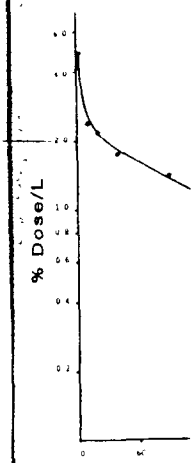

FIG. 1. Disappear
sulfate plotted as
blood sampling in

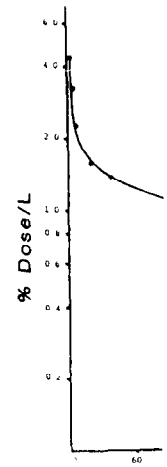

FIG. 2. Disappea
sulfate plotted a
blood sampling in

where A′ and B′ are the intercepts on the ordinate axis obtained by extrapolation of the two curves to zero time (expressed as the fraction of the administered dose of radioactivity per liter plasma), α is the slope of the first component, and β is the slope of the final part of the curve. The inner volume (V_1), the volume into which the IV injected hormone was immediately distributed, was calculated from the relationship $V_1 = 1/(A' + B')$ at time zero. The rate constant (k) of total removal (reversible and irreversible) of equilin sulfate from V_1 was calculated using the equation (21): $k = (A'\alpha + B'\beta)/(A' + B')$.

The percentage of equilin sulfate removed irreversibly was calculated from the expression (21): rate of irreversible removal/rate of total removal = MCR/kV_1.

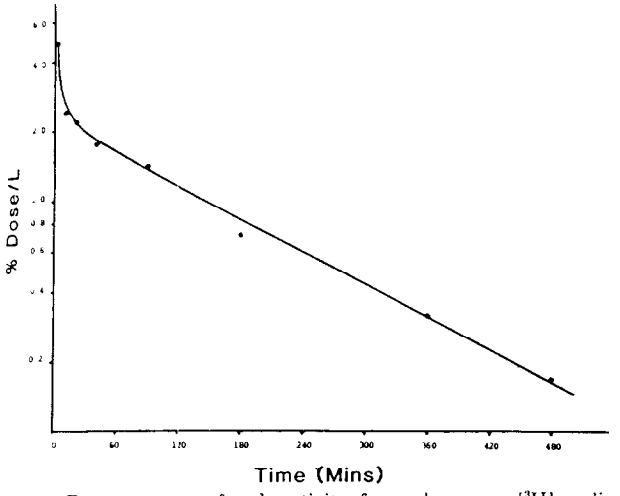

FIG 1. Disappearance of radioactivity from plasma as [^{3}H]equilin sulfate plotted as a percentage of the administered dose *vs* time of blood sampling in female subject 4

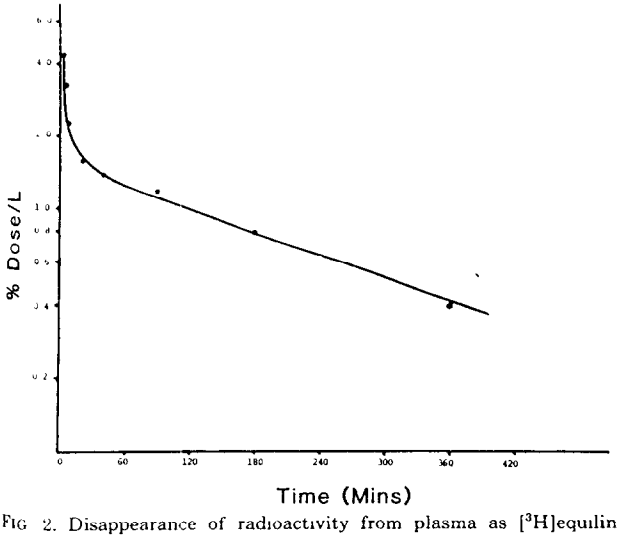

FIG 2. Disappearance of radioactivity from plasma as [^{3}H]equilin sulfate plotted as a percentage of the administered dose *vs.* time of blood sampling in male subject 5

Results

Pharmacokinetics of equilin sulfate

The concentration of radioactivity as equilin sulfate, expressed as a percentage of the administered dose, was plotted on a semilog scale (y-axis) against time (x-axis). In both female and male subjects, the disappearance of radioactivity could be expressed as a function which is the sum of two exponentials. Two such plots are shown in Figs. 1 and 2. The data obtained from the analysis of each individual disappearance curve are shown in Table 2. The slopes and intercepts from these five studies were combined, and the mean slopes and intercepts are shown in Fig. 3. The mean MCR was found to be 315 ± 73 (±SEM) liters/day; when corrected for surface area, the mean was 176 ± 44 (±SEM) liters/day·m^2.

The mean value for the initial volume of distribution (V_1) was 12.4 ± 1.6 liters, and the disappearance of equilin sulfate from this inner volume had a mean half-life of 5 ± 1.2 min. The mean half-life of the slower component of equilin sulfate was 190 ± 23 min (Table 2).

The mean rate constant (k) (21) of total removal (reversible and irreversible) of equilin sulfate from V_1 was 163 ± 19 U/day. The fraction of equilin sulfate removed irreversibly (MCR/kV_1) was calculated to be 0.158 ± 0.02 (Table 2).

MCR of equilin

The MCR of equilin was measured in one postmenopausal woman and one normal man by injecting [^{3}H] equilin. The disappearance curve of radioactive equilin could be fitted by a single straight line, consistent with a one-compartment model described by a single y-intercept (A′) and a single slope (α), as shown in Figs. 4 and 5. The MCR of equilin in the postmenopausal woman and man was calculated to be 5280 and 3944 liters/day or 3300 and 1982 liters/day·m^2, respectively. The V_1 values were 100 and 62.5 liters (Table 3), and the disappearance of equilin from this inner volume had half-lives of 27 and 19 min in the postmenopausal woman and man, respectively (Table 3).

Formation and kinetics of equilenin, 17β-dihydroequilin, and 17β-dihydroequilenin sulfates after a bolus injection of [^{3}H]equilin sulfate

The appearance of radioactivity in plasma specifically as equilenin, 17β-dihydroequilin, and 17β-dihydroequilenin sulfates after the administration of [^{3}H]equilin sulfate was measured in four postmenopausal women, and the results were expressed as percent dose per liter and are summarized in Tables 4–6. These three metabolites were isolated only from the sulfate fraction. Equi-

TABLE 2 Data calculated from the analysis of the equilin sulfate plasma concentration curves

Subject no	Sex and age (yr)	Dose of [^{3}H] equilin sulfate (dpm × 10^6)	Fraction of dose		α (U/day)	β (U/day)	k (U/day)	V_1 (liters)	$t_{1/2}$ (min)		MCR (liters/day)	MCR (liters/day·m^2)	MCR/kV_1
			A'	B'					Component 1	Component 2			
1	F, 38	96	0 10	0 0150	103.2	3 00	90.1	8.7	9.8	170	168	93	0.215
2	F, 60	120	0 05	0 0054	230.3	3.60	208.2	18.0	4.0	270	582	342	0.155
3	F, 57	90	0.06	0.020	242.8	4.16	179.2	13 3	4.4	180	199	117	0 083
4	F, 51	114	0.06	0.022	248.7	7 2	170 0	11.4	4.1	150	303	168	0.156
5	M, 58	92	0 04	0.016	231.8	5.47	167.0	10.8	4.0	180	323	162	0.179
Mean ± SEM			0 06 ± 0 01	0 016 ± 0.003	211 ± 27	4.7 ± 0 75	163 ± 19	12 4 ± 1 6	5.2 ± 1.2	190 ± 23	315 ± 73	176 ± 44	0.158 ± 0.02

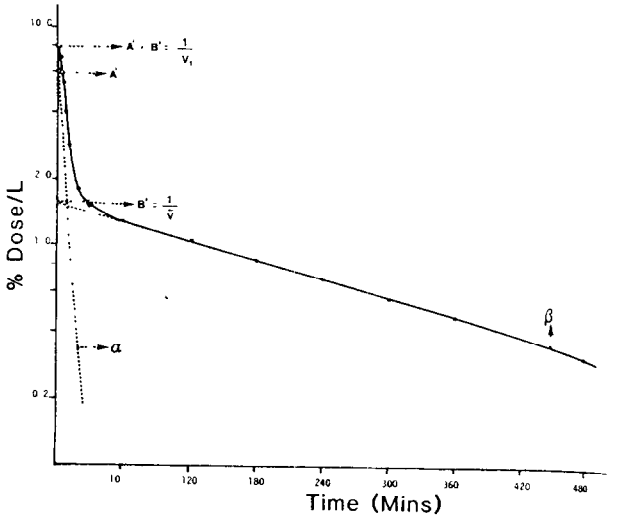

FIG. 3. Mean disappearance of radioactivity from plasma as [^{3}H] equilin sulfate plotted as a percentage of the administered dose *vs* time of blood sampling in subjects 1–5. The slopes and intercepts from the five studies were combined, and the mean slopes and intercepts are shown.

lenin sulfate was formed very rapidly, with the maximum concentration being present in the first blood sample taken 3 min after the administration of equilin sulfate (Table 4 and Fig. 6). The disappearance of equilenin sulfate was characterized by two components similar to those observed for equilin sulfate (Fig. 1 and 2), with the half-life of the first component being approximately 3 min. In contrast, the formation of 17β-dihydroequilin and 17β-dihydroequilenin sulfates increased with time (Tables 5 and 6) and reached maximum concentrations by 20 min. The levels of the three metabolites gradually decreased with time, and 8 h after the injection of equilin sulfate, they were barely detectable. In Fig. 6, the mean levels of the above three metabolites isolated from the plasma of the four subjects are plotted as the percent dose formed per liter against time.

The 17α-reduced metabolites, such as 17α-dihydroequilin and 17α-dihydroequilenin, were searched for by careful analysis of the areas on paper chromatograms corresponding in mobility to the above two steroids. These were devoid of any radioactivity, indicating that the [^{3}H]equilin sulfate was metabolized to only labeled equilenin sulfate, 17β-dihydroequilin sulfate, and 17β-dihydroequilenin sulfate.

Formation and kinetics of equilin sulfate and 17β-dihydroequilin sulfate and 17β-dihydroequilin after a bolus injection of [^{3}H]equilin

The appearance of radioactivity specifically as equilin sulfate, 17β-dihydroequilin sulfate, and 17β-dihydro-

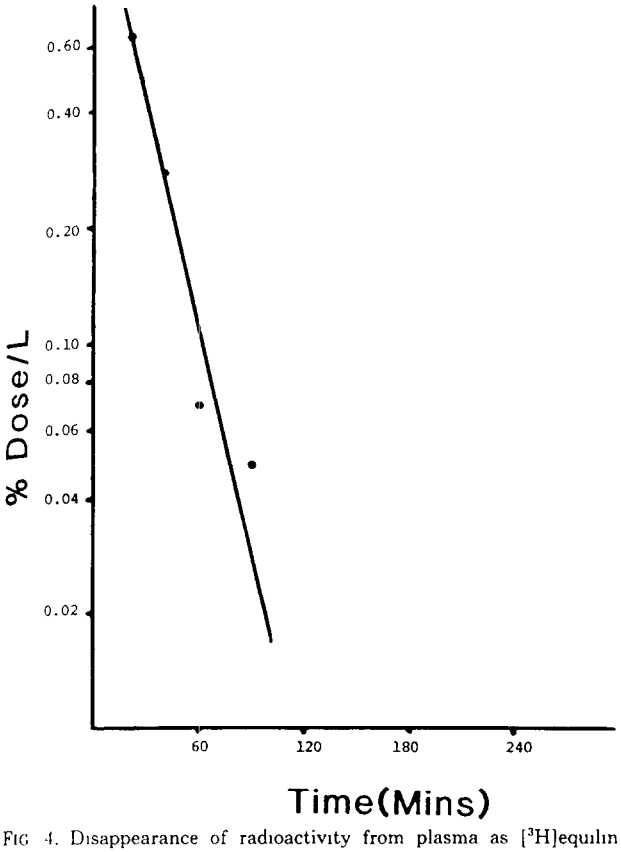

FIG. 4. Disappearance of radioactivity from plasma as [^{3}H]equilin plotted as a percentage of the administered dose *vs* time of blood sampling in male subject 5

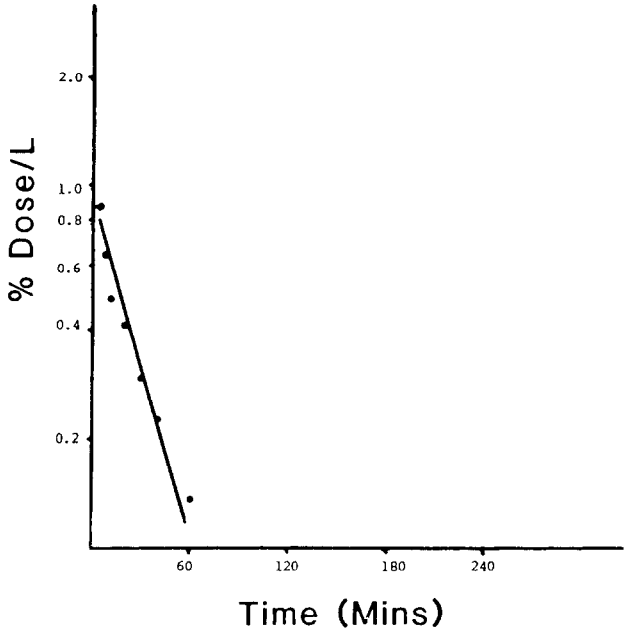

FIG. 5 Disappearance of radioactivity from plasma as [^{3}H]equilin plotted as a percentage of the administered dose *vs.* time of blood sampling in female subject 6

equilin during the first 90 min in plasma after the administration of [^{3}H]equilin to a postmenopausal woman (subject 1, Table 3) is summarized in Table 7. The rate of formation of both sulfate-conjugated estrogens increased with time, reached maximum levels 40 min after the bolus injection of equilin, and then very gradually began to decline. The formation of 17β-dihydroequilin reached maximum levels by 20 min and then declined fairly rapidly, as shown in Table 7. In the subject discussed above, the [^{3}H]equilin injected disappeared from plasma very rapidly (Fig. 5) and was not detectable 60 minutes after its administration. In contrast, at 30 min the concentration of equilin sulfate (Table 7) exceeded the highest levels of equilin found in the first sample taken at 3 min (Fig. 5). These results indicate that equilin is very rapidly metabolized to equilin sulfate, which appears to be the main circulating metabolite of equilin.

Discussion

Equilin sulfate is one of the main components of conjugated equine estrogen preparations (such as Premarin) which have been used for over 3 decades, but very little is known about the metabolic fate of this and other ring B unsaturated estrogens in the human. In this investigation, the *in vivo* metabolism of equilin sulfate and equilin was investigated in postmenopausal women and a normal man.

The MCR of [^{3}H]equilin sulfate or [^{3}H]equilin was calculated by analysis of plasma samples taken after rapid injection of [^{3}H]equilin sulfate or [^{3}H]equilin. The data points relating to the disappearance of equilin sulfate and equilin from plasma suggested that a two-compartment model can be used for the analysis of the MCR of equilin sulfate and a single compartment model can be used for equilin (19–21).

The mean MCR of equilin sulfate was calculated to be 176 ± 44 liters/day·m^2 for women and men. This value is only 6–8% of that calculated for the unconjugated equilin (3300 and 1982 liters/day·m^2). A similar relationship between the MCR of the sulfate and that of the unconjugated steroid has been noted for estrone (8), dehydroisoandrosterone, and testosterone (25, 26).

Using a two-compartment model, Longcope (8) reported the MCR of estrone sulfate in males to be 105 ± 20 liters/day·m^2 (range, 60–205) by the single injection technique and 80 ± 10 liters/day·m^2 (range, 45–115) by the constant infusion technique. Ruder *et al.* (9), using a single compartment model, calculated the MCR of estrone sulfate to be 87 ± 36 liters/day·m^2 (range, 46–163)

TABLE 3 Data calculated from the analysis of the plasma concentration curves

Subject no	Sex and age (yr)	Dose of [^{3}H]equilin (dpm × 10^6)	A′ fraction of dose	α (U/day)	V_1 (liters)	$t_{1/2}$ (min)	MCR (liters/day)	MCR (liters/day m^2)
5	M, 58	126	0.016	63.1	62.5	19	3944	1982
6	F, 49	74	0.010	52.8	100	27	5280	3300

TABLE 4. Kinetics of equilenin sulfate formation after a bolus injection of [^{3}H]equilin sulfate

Subject no.	Sex and age (yr)	Dose [^{3}H]equilin sulfate (dpm × 10^6)	Plasma [^{3}H]equilenin sulfate (% dose/liter)								
			3 min	6 min	10 min	20 min	40 min	90 min	180 min	360 min	480 min
4	F, 38	96	2.8	1.6	1.0	0.54	0.50	0.47	0.3	0.27	0.2
5	F, 60	120	1.3	0 6	0.5	0.44	0.40	0.38	0.24	0.11	0.07
6	F, 57	90	1.1	0.7	0.6	0.8	1.0	0.6	0.4	0.3	0.3
7	F, 51	114	1.73	1 60	0.8	0.70	0.6	0.4	0.3	0.2	0.1
Mean ± SEM			1 7 ± 0.38	1.1 ± 0.28	0.7 ± 0.11	0.6 ± 0.08	0.6 ± 0.1	0.5 ± 0.05	0.3 ± 0 03	0.2 ± 0.04	0.16 ± 0.05

TABLE 5 Kinetics of [^{3}H]17β-dihydroequilin sulfate formation after a bolus injection of [^{3}H]equilin sulfate

Subject no	Sex and age (yr)	Dose of [^{3}H] equilin sulfate (dpm × 10^6)	Plasma [^{3}H]17β-dihydroequilin sulfate (% dose/liter)								
			3 min	6 min	10 min	20 min	40 min	90 min	180 min	360 min	480 min
4	F, 38	96	0.09	0.3	0.4	0.5	0 4	0.3	0.2	0.2	0.2
5	F, 60	120	0.1	0.2	0.3	0 3	0.2	0.2	0.1	0.05	0.05
6	F, 57	90	0.2	0.4	0.6	0.4	0.4				
7	F, 51	114	0.2	0.2	0.4	0.5	0.4	0.3	0.2	0.01	0 05
Mean ± SEM			0.15 ± 0.03	0.28 ± 0.05	0.42 ± 0.06	0.42 ± 0.05	0.35 ± 0.05	0.26 ± 0.03	0.16 ± 0.03	0 12 ± 0.04	0.1 ± 0.05

TABLE 6 Kinetics of [^{3}H]17β-dihydroequilenin sulfate formation after a bolus injection of [^{3}H]equilin sulfate

Subject no	Sex and age (yr)	Dose [^{3}H] equilin sulfate (dpm × 10^6)	Plasma [^{3}H]17β-dihydroequilenin sulfate (% dose/liter)								
			3 min	6 min	10 min	20 min	40 min	90 min	180 min	360 min	480 min
4	F 38	96	0 17	0.14	0 19	0 23	0.20	0.16	0 14	0 14	0 12
5	F, 60	120	0 15	0.08	0 11	0 16	0 14	0 13	0 07	0.05	0.05
6	F, 57	90	0.13	0 18	0 21	0 22	0.24				
7	F, 51	114	0.12	0 19	0.19	0 19	0.20	0.14	0.11	0 09	0.05
Mean ± SEM			0 14 ±0 01	0.15 ± 0 02	0.18 ± 0.02	0 20 ± 0.01	0.19 ± 0 02	0.14 ± 0.01	0.10 ± 0 02	0.09 ± 0.02	0 07 ± 0.02

for males and 94.1 ± 22 liters/day·m^2 (range, 39–141) for females. The androgen sulfates, such as dehydroisoandrosterone and testosterone, have a much lower (7.7, 21.5 liters/day) MCR (25, 26). Our results with equilin sulfate indicate that this compound is cleared from the human body faster than any of the above steroid sulfates and that there is no sex difference.

The very low MCR of the androgen sulfates may be due to plasma protein binding of the sulfated steroid (8, 26). It has also been reported that estrone sulfate and estradiol sulfate bind to human serum albumin quite strongly (27, 28). Whether the low MCR of equilin sulfate, in contrast to that of an unconjugated equilin, is due to its binding with human serum albumin (and perhaps other blood proteins) or to the ionic nature of the steroid sulfates remains to be established.

The low MCR can also result if only a small fraction of equilin sulfate is being metabolized. While our results indicate that equilin sulfate is metabolized to sulfate esters of equilenin, 17β-dihydroequilin, and 17β-dihydroequilenin, this metabolism was determined after a single injection of [^{3}H]equilin sulfate, and it appears that only a small fraction was metabolized (15.8%). More quantitative experiments under steady state conditions, such as constant infusions (19, 20), will be necessary to resolve this question.

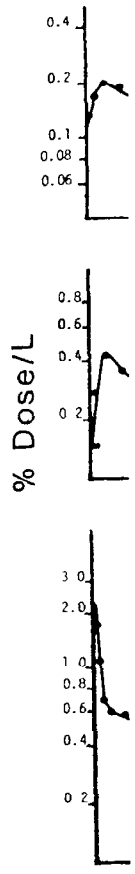

FIG 6. Formation
dihydroequilenin s
to postmenopausal
dihydroequilin; 17

TABLE 7. Plasma
injection of [^{3}H]e

Metabolite isolated
Equilin sulfate
17β-Dihydroequilin sulfate
17β-Dihydroequilin

The MCR
965 liters/day
significant dif
males. Simila
600–790 liters

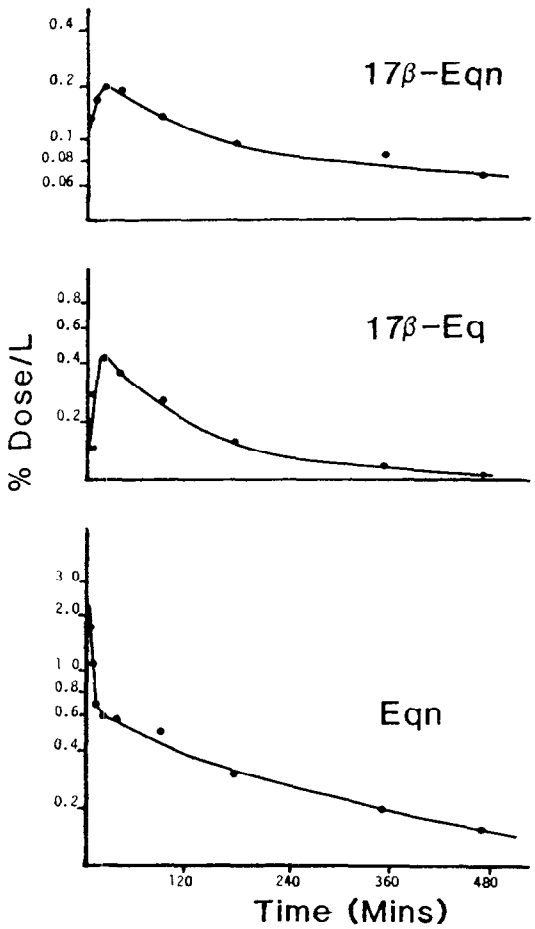

FIG. 6 Formation kinetics of equilenin, 17β-dihydroequilin, and 17β-dihydroequilenin sulfates after the administration of [^{3}H]equilin sulfate to postmenopausal women (subjects 1–4). Eqn, Equilenin; 17β-Eq, 17β-dihydroequilin; 17β-Eqn, 17β-dihydroequilenin.

TABLE 7. Plasma levels of ^{3}H-labeled metabolites formed after a bolus injection of [^{3}H]equilin to a postmenopausal woman

Metabolite isolated	Plasma levels (% dose/liter)							
	6 min	8 min	10 min	20 min	30 min	40 min	60 min	90 min
Equilin sulfate	0.30	0.40	0.60	0.70	1.40	1.60	1.40	1.20
17β-Dihydroequilin sulfate	0.05	0.05	0.08	0.10	0.18	0.23	0.20	0.17
17β-Dihydroequilin	0.20	0.20	0.23	0.26	0.21	0.13	0.12	0.07

The MCR of estrone has been reported to be around 965 liters/day·m^2 (30) and 1310 liters/day·m^2 (29). No significant difference was noted between males and females. Similarly, the MCR of estradiol was found to be 600–790 liters/day·m^2 for females (29, 31) and 830–990 liters/day·m^2 for males (29, 31). In contrast, the MCRs of equilin determined in one male and one female were found to be 1982 and 3300 liters/day·m^2, respectively, values that are approximately 2–5 times higher than those found for estrone and estradiol. These results indicate that equilin is being cleared much more rapidly than either estrone or estradiol. It appears from our preliminary results that equilin is rapidly metabolized to equilin sulfate, 17β-dihydroequilin sulfate, and 17β-dihydroequilin, with equilin sulfate being the major metabolite in the circulation.

The amount of radioactive material present in the unconjugated fraction of plasma after the administration of equilin sulfate was not sufficient for further analysis; however, we have reported (18) that after the administration of Premarin to postmenopausal women, some of the equilin sulfate present in Premarin appears quickly in the serum as unconjugated equilin. These and the present results indicate that equilin sulfate is the main circulating form from which small amounts of unconjugated equilin are being released. A similar relationship between estrone and estrone sulfate has been described (9).

The half-life of equilin was found to be between 19–27 min, and in contrast, the main slower (second) component of equilin sulfate has a half-life of 190 min (3.2 h). This half-life is approximately 2–3 times lower than that reported for estrone sulfate [5.3–9 h (9)].

The V_1 for equilin sulfate is much larger than the plasma volume. This suggests that equilin sulfate may not be strongly bound to any plasma protein. The disappearance of equilin sulfate from the inner volume has a mean half-life of 5.2 ± 1.2 min, which is similar to that reported for estrone sulfate [V_1 = 7.2 liters; $t_{1/2}$, 3 min (8)]. The mean value for k (21), the rate constant of total removal (reversible plus irreversible) of equilin sulfate from the circulation, ranged from 90.1–208 U/day. The ratio of irreversible over total removal (MCR/kV_1) had a mean value of 0.158 ± 0.02, indicating that 15.8% of the equilin sulfate leaving the circulation is lost irreversibly, and is compatible with the observed low MCR for equilin sulfate. This is a relatively small fraction metabolized compared to this fraction for other steroids (32–34), but is similar to that reported for estrone sulfate [11.1% (8)].

In the present investigations, the formation of equilenin sulfate, 17β-dihydroequilenin sulfate, and 17β-dihydroequilin sulfate from [^{3}H]equilin sulfate has been demonstrated. Similarly, the formation of equilin sulfate, 17β-dihydroequilin sulfate, and 17β-dihydroequilin from [^{3}H]equilin was demonstrated. No 17α-reduced metabolites of equilin or equilin sulfate were isolated. These findings are similar to the conversion of estrone to 17β-estradiol. Recently, we reported that after the adminis-

tration of equilin to normal men, 17β-dihydroequilin, 17β-dihydroequilenin, and equilenin were isolated from the urine (35). The bulk of these metabolites present in urine were conjugated to glucuronic acid. In contrast, in the present study, no glucuronide conjugates were detectable in the plasma, where the main circulating form of equilin and its metabolites are in the form of sulfate conjugates. Thus, before excretion, these circulating sulfate conjugates are hydrolyzed and then reconjugated with glucuronic acid. The formation of the 17β-dihydroequilin and its sulfate is of major importance, as it has been shown that this estrogen is 8 times more potent as a uterotropic agent than the parent compound equilin sulfate (36). Further studies are required to determine more precisely the amount of 17β-dihydroequilin formed and its metabolic fate in the human.

Acknowledgments

We thank Drs. A F. Clark and C. E. Bird (Queen's University, Kingston, Ontario, Canada) for their helpful discussions

References

1. Herr F, Revesz C, Manson AJ, Jewell JB 1970 Biological properties of estrogen sulfate. In Berstein S, Solomon S (eds) Chemical and Biological Aspects of Steroid Conjugation. Springer-Verlag, New York, p 368
2. Johnson R, Masserono R, Haring R, Kho B, Schivling G 1975 Quantitative GLC determinations of conjugated estrogens in raw materials and printed dosage forms. J Pharm Sci 64:1007
3. Smith DC, Prentice R, Thompson DJ, Hermann L 1975 Association of exogenous estrogen and endometrial carcinoma. N Engl J Med 293:1164
4. Ziel HK, Finkle WD 1975 Increased risk of endometrial carcinoma among users of conjugated estrogens. N Engl J Med 293:1167
5. Gordon J, Regan JW, Finkle WD, Ziel HK 1977 Estrogen and endometrial carcinoma an independent pathology review supporting typical risk estimate N Engl J Med 297:570
6. Twombly GH, Levitz M 1960 Metabolism of estrone ^{14}C-16-sulfate in women. Am J Obstet Gynecol 80:889
7. Jirku H, Levitz M 1969 Biliary and urinary metabolites of estrone-6, 7-sulfate-^{35}S in women J Clin Endocrinol Metab 29:615
8. Longcope C 1972 The metabolism of estrone sulfate in normal males. J Clin Endocrinol Metab 34:113
9. Ruder HJ, Loriaux DL, Lipsett MB 1972 Estrone sulfate production rate and metabolism in man J Clin Invest 51:1020
10. Loriaux DL, Ruder HJ, Lipsett MB 1971 The measurement of estrone sulfate in plasma. Steroids 18:463
11. Bhavnani BR, Short RV, Solomon S 1969 Formation of estrogens by the pregnant mare. Metabolism of 7-^{3}H-dehydroisoandrosterone and 4-^{14}C androstenedione injected into the umbilical vein Endocrinology 85:1172
12. Bhavnani BR, Short RV, Solomon SS 1971 Formation of estrogens by the pregnant mare II. Metabolism of ^{14}C-acetate and ^{3}H-cholesterol injected into the fetal circulation. Endocrinology 89 1152
13. Bhavnani BR, Short RV 1973 Formation of steroids by the pregnant mare III Metabolism of ^{14}C-squalene and ^{3}H-dehydroisoandrosterone injected into the fetal circulation. Endocrinology 92:657
14. Bhavnani BR, Short RV 1973 Formation of steroids by the pregnant mare. IV. Metabolism of ^{14}C-mevalonic acid and ^{3}H-dehydroisoandrosterone injected into the fetal circulation. Endocrinology 92:1397
15. Bhavnani BR, Martin LJ, Baker RD 1975 Formation of steroids by the pregnant mare. V. Metabolism of ^{14}C-isopentenylpyrophosphate and ^{3}H-dehydroisoandrosterone injected into the fetus. Endocrinology 96:1009
16. Bhavnani BR, Woolever CA 1978 Formation of steroids by the pregnant mare. VI. Metabolism of ^{14}C-farnesylpyrophosphate and ^{3}H-dehydroepiandrosterone injected into the fetus Endocrinology 103:2291
17. Bhavnani BR, Woolever CA 1981 *In vivo* metabolism of [^{3}H]equilin in the pregnant mare Endocrinology 108:232
18. Bhavnani BR, Sarda IR, Woolever CA 1981 Radioimmunoassay of plasma equilin and estrone in postmenopausal women following administration of Premarin. J Clin Endocrinol Metab 52:741
19. Tait JF, Tait SAS, Little B, Laumas KR 1961 The disappearance of 7-^{3}H-α-aldosterone in the plasma of normal subjects. J Clin Invest 40:72
20. Tait JF, Burstein S 1964 *In vivo* studies of steroid dynamics in man. In Pincus G, Thimann KV, Astwood EB (eds). Steroid Dynamics. Academic Press, New York, p 441
21. Rizkallah T, Gurpide E, VandeWiele RL 1969 Metabolism of HCG in man. J Clin Endocrinol Metab 29:92
22. Nehr R 1964 Steroid Chromatography. Elsevier, New York, p 134
23. Bradlow HL 1968 Extraction of steroid conjugates with a neutral resin. Steroids 11:265
24. Bhavnani BR, Solomon S 1969 Preparation of 16α-hydroxypregnenolone-3-sulfate and a study of its anomalous behaviour during hydrolysis. Endocrinology 84:1230
25. Sandberg E, Gurpide E, Lieberman S 1964 Quantitative studies on the metabolism of dehydroisoandrosterone sulfate. Biochemistry 3:1256
26. Wang DY, Bulbrook RD, Sneedon A, Hamilton T 1967 The metabolic clearance rates of dehydroepiandrosterone, testosterone and their sulphate esters in man, rat and rabbit. J Endocrinol 38:307
27. Rosenthal HE, Pietrzak E, Slaunwhite Jr WR, Sandberg AA 1972 Binding of estrone sulfate in human plasma J Clin Endocrinol Metab 34:805
28. Rosenthal HE, Ludwig GA, Pietrazak E, Sandberg AA 1975 Binding of the sulfate of estradiol-17β to human serum albumin and plasma. J Clin Endocrinol Metab 41:1144
29. Longcope C, Layne DS, Tait JF 1968 Metabolic clearance rates and interconversions of estrone and 17β-estradiol in normal males and females. J Clin Invest 47:93
30. Longcope C, Tait JF 1971 Validity of metabolic clearance and interconversion rates of estrone and 17β-estradiol in normal adults. J Clin Endocrinol Metab 32:481
31. Hembree WC, Bardin CW, Lipset MB 1969 A study of estrogen metabolic clearance rates and transfer factors. J Clin Invest 48:1809
32. Tait JF, Tait SAS, Little B, Laumans KR 1961 The disappearance of 7-^{3}H-α-aldosterone in the plasma of normal subjects. J Clin Invest 40:72
33. Southren AL, Tochimoto S, Carbody NC, Isurgui K 1965 Plasma production rates of testosterone in normal adult men and women and in patients with the syndrome of feminizing testes. J Clin Endocrinol Metab 25:1441
34. Flood C, Pratt H, Longcope C 1976 The metabolic clearance and blood production rates of estriol in normal, non-pregnant women. J Clin Endocrinol Metab 42:1
35. Bhavnani BR, Woolever CA 1981 The metabolism of equilin in normal men. J Steroid Biochem 17:217
36. Dorfman RI, Dorfman AS 1954 Estrogen assays using the rat uterus. Endocrinology 55:65

0021-972X/83/5605-1
Journal of Clinical E
Copyright © 1983 by T

Steroid Adrena

J. IAN MAS(

The Cecil H. an and Obstetrics-(

ABSTRA
midtimes
ACTH Po
and LH di
The princ
cells were
nenolone
productio
progestero
methyl-1
droxyster
caused on
confirmin

THE CA
crete la
essary for n
ognized. Nea
200 mg ster
techniques h
human fetal
stimuli. The
(3, 4) and c
In long term
istics more
cells. In part
sis, or decre
hydrogenase
creased synt
(F) (3–6).
more impor
effect of hc
nesis, we us
zone of hun
using simil

Received S
Address all
Mason, Cecil
Sciences, The
Hines Boulev.
* This wor
and DFG Gra

DEPARTMENT OF HEALTH & HUMAN SERVICES

Office of Inspector General

Memorandum

Date MAY 16 1997

From June Gibbs Brown
Inspector General

Subject Review of the Food and Drug Administration's Handling of Issues Related to Conjugated Estrogens (A-15-96-50002)

To Michael A. Friedman, M.D.
Lead Deputy Commissioner
Food and Drug Administration

The attached report provides the results of our review of the Food and Drug Administration's (FDA) handling of issues related to conjugated estrogens (Premarin), a commonly prescribed product for menopausal symptoms and the prevention and management of osteoporosis. This review was performed at the request of the Chairman, Subcommittee on Oversight and Investigations, House Committee on Commerce.

We were specifically asked to respond to questions regarding: whether unapproved formulations of Premarin are being marketed by its manufacturer, Wyeth-Ayerst; whether data exists showing that the currently marketed version of Premarin is safe and effective compared to an earlier version tested in the 1970's; the basis upon which FDA approved a new Premarin-based product called Prempro; and FDA's processing of a Wyeth-Ayerst citizen petition requesting the agency to recognize a third active ingredient in Premarin and not approve any generic versions lacking the third ingredient.

If you have any questions or comments regarding the issues discussed in this report, please call me or have your staff contact Joseph J. Green, Assistant Inspector General for Public Health Service Audits, at (301) 443-3582.

Attachment

Department of Health and Human Services

OFFICE OF INSPECTOR GENERAL

REVIEW OF THE FOOD AND DRUG ADMINISTRATION'S HANDLING OF ISSUES RELATED TO CONJUGATED ESTROGENS

JUNE GIBBS BROWN
Inspector General

MAY 1997
A-15-96-50002

EXECUTIVE SUMMARY

BACKGROUND

The Food and Drug Administration (FDA) is the Federal agency responsible for approving applications to market new drugs, new indications for already marketed drugs, and generic versions of brand name drugs. Conjugated estrogens products represent a class of marketed drugs used primarily for the treatment of menopausal symptoms in women and for the prevention and management of osteoporosis, a crippling disease that causes thinning of the bone. Wyeth-Ayerst is a drug manufacturer that markets: (1) Premarin, approved in 1942, which is made from the urine of pregnant mares and is the Nation's only conjugated estrogens product; and (2) Prempro, approved in 1994, which combines Premarin and another hormone called progestin. At this time, there are no approved applications to market generic versions of these brand name drugs.

Once a drug is approved, drug manufacturers are required to obtain FDA's approval before adding or deleting an ingredient, or otherwise changing the composition of a drug product, other than deletion of an ingredient intended only to affect the color of the drug product. Current regulations require that when there is a change in the manufacturing process, including a change in product formulation or dosage strength, beyond the variations provided for in the approved application, drug manufacturers are required to show that any reformulations are bioequivalent[1] to the approved product.

The FDA uses a process called the citizen petition to allow anyone--individuals or companies--to request the agency to make changes to its regulations. In November 1994, Wyeth-Ayerst submitted a citizen petition requesting FDA to: (1) recognize an ingredient of Premarin--delta 8,9 (dehydroestrone sulfate (DHES))--as an essential (but not an active[2]) ingredient in Premarin; and (2) not approve any generic version of Premarin that does not contain DHES. The firm amended its petition in December 1996 to request FDA to recognize DHES as an active ingredient. To date, FDA has not made a decision as to whether to approve Wyeth-Ayerst's citizen petition request. However, on May 5, 1997, FDA's Director, Center for Drug Evaluation and Research (CDER), announced that, because the active ingredients of Premarin have not been adequately defined, the agency could not at this time approve generic applications for synthetic versions of conjugated estrogens. The FDA had been reviewing two such applications since 1994 and 1995, respectively.

The House Committee on Commerce, Subcommittee on Oversight and Investigations, has raised concerns that Wyeth-Ayerst may have made misrepresentations in its submissions to

---

[1] To show bioequivalence between reformulated drugs, there must not be a significant difference in the rate and extent to which the active ingredient of each product becomes available at the site of drug action.

[2] An active ingredient is any component that is intended to furnish pharmacologic activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease.

FDA regarding Premarin, and that FDA may have failed to adequately review such submissions. The Subcommittee requested the Office of Inspector General (OIG) in July 1996 to answer specific questions regarding: Premarin, another Wyeth-Ayerst product called Prempro, the citizen petition related to Premarin, and generic versions of Premarin.

OBJECTIVE

Our objective was to answer the Subcommittee's questions in the following three areas:

1. Premarin: The questions focused on possible unapproved reformulations; whether the reformulations were bioequivalent; and the basis on which FDA allowed the continued marketing of Premarin.

2. Prempro: The question focused on the basis on which Wyeth-Ayerst won approval for Prempro's use in the prevention and management of osteoporosis.

3. Wyeth-Ayerst's citizen petition regarding Premarin and generic versions of Premarin: The questions focused on FDA's examination of data and claims made in Wyeth-Ayerst's citizen petition and the agency's handling of brand name and generic versions of Premarin.

SUMMARY OF FINDINGS

Premarin: According to FDA, there have been no unapproved formulations of Premarin. Regarding the issue of bioequivalency among the formulations, however, we found that FDA does not have evidence demonstrating that the currently marketed formulation of Premarin is bioequivalent to the version tested for osteoporosis in the late 1970's. This is because no in vivo (i.e., in the living body) bioequivalence requirement was in effect for conjugated estrogens at that time. Concerned about lack of bioequivalency data and the continued safety and effectiveness of Premarin, FDA in 1993 directed Wyeth-Ayerst to conduct a new dose-ranging study of the drug. As of January 1997, 818 women, or about 30 percent of the total planned enrollment of 2,688, have entered into the multi-year study.

Prempro: The Premarin tablet formulation used in the combination drug Prempro (Premarin/medroxyprogesterone acetate) slightly differed from the marketed Premarin, but Wyeth-Ayerst submitted in vivo bioequivalence data to demonstrate that the new and currently marketed formulations were bioequivalent.

Citizen Petition and Generic Versions of Premarin: The FDA is in the process of reviewing the claims and data associated with Wyeth-Ayerst's citizen petition, which was submitted to the agency over 2 years ago. The FDA has thus far found deficiencies in the design of studies submitted to support Wyeth-Ayerst's claims, but no misrepresentations in data in the firm's studies have been identified. Regarding the Subcommittee's concern that FDA may have held generic drug firms to a higher standard than the brand-name maker of Premarin,

Wyeth-Ayerst, we noted that the agency was also concerned about possible differing standards in terms of bioequivalency requirements for the generic and brand name versions. However, upon further investigation, FDA determined there were no unapproved reformulations of the brand name Premarin that would have required Wyeth-Ayerst to submit additional bioequivalency data.

Beyond the Subcommittee's specific questions, we identified other concerns regarding the citizen petition process--namely that the process has been extended for an excessive period of time in the Wyeth-Ayerst case; and FDA does not have policies and procedures governing such an important process, one which can impact the marketability of generic versions of Premarin.

TABLE OF CONTENTS

INTRODUCTION

BACKGROUND

FDA ROLE AND ORGANIZATION

The FDA is the Federal agency responsible for approving applications to market new drugs, new indications for already marketed drugs, and generic versions of brand name drugs. Since 1962, sponsors have been required to demonstrate to FDA that their drug is both safe and effective. Prior to 1962, there was only the requirement to show the drug was safe.

Once a drug is approved, drug manufacturers are required to obtain FDA's approval before adding or deleting an ingredient, or otherwise changing the composition of a drug product, other than deletion of an ingredient intended only to affect the color of the drug product. Current regulations require that when there is a change in the manufacturing process, including a change in product formulation or dosage strength, beyond the variations provided for in the approved application, drug manufacturers are required to show that any reformulations are bioequivalent to the approved product.

Various organizations within FDA are responsible for handling issues regarding brand name and generic drugs:

- The FDA's CDER is the organization that handles drug issues. One of CDER's chief responsibilities is reviewing new drug applications (NDAs) that sponsors submit to seek approval for marketing new drug products. Within CDER, 5 offices of drug evaluation oversee 15 divisions that review NDAs for new brand name prescription drugs. For conjugated estrogens, the Division of Metabolism and Endocrine Drug Products was responsible for handling Premarin until 1996 when responsibility was transferred to the newly formed Division of Reproductive and Urologic Drug Products.

- The CDER's Office of Generic Drugs (OGD) is responsible for processing applications from sponsors seeking to market generic versions of brand name drugs.

- The CDER's Office of Compliance, Division of Scientific Investigations, is responsible for directing and coordinating on-site inspections of sponsors and investigations of preclinical and clinical drug product studies.

- The FDA's Office of Regulatory Affairs (ORA) directs the agency's field staff which, among other duties, performs inspections of regulated firms. The ORA conducts routine inspections according to a pre-determined schedule; and for-cause inspections, which are requested for specific reasons. The ORA also oversees the implementation of the policy entitled, "Fraud, Untrue Statements of Material Facts, Bribery, and Illegal Gratuities," (also known as FDA's Fraud Policy), published in the Federal

Register in 1991 to address instances when misrepresentations are suspected in submissions to the agency.

To augment FDA's decisionmaking processes, the agency routinely uses advisory committees to provide scientific input to its product approval processes. To address various issues regarding conjugated estrogens, FDA has over the years convened the Obstetrics and Gynecology Advisory Committee, the Fertility and Maternal Health Drugs Advisory Committee, the Endocrinologic and Metabolic Drugs Advisory Committee, and the Generic Drugs Advisory Committee.

CONJUGATED ESTROGENS

Conjugated estrogens products represent a class of marketed drugs used primarily for the treatment of female menopausal symptoms, such as hot flashes, and for the prevention and management of osteoporosis, a crippling disease that causes thinning of the bone. Estrogens are important in the development and maintenance of the female reproductive system and secondary sex characteristics. They also contribute to the shaping of the skeleton.

Premarin, manufactured by Wyeth-Ayerst from the urine of pregnant mares, is the only brand of conjugated estrogens sold in the United States, with over 8 million women taking it daily. The FDA first approved Premarin in 1942 as safe for its intended use in the treatment of various menopausal symptoms; and in 1986, it deemed Premarin and the entire class of short-acting estrogens[3] as effective for the prevention and management of osteoporosis.

In 1994, FDA approved Wyeth-Ayerst's new drug application to market Prempro, a drug combining Premarin and another hormone--progestin. Studies have shown that the introduction of progestin lowers the incidence of endometrial cancer for the woman whose uterus is intact. Premarin continues to be prescribed for the woman who has had her uterus removed.

DRUG EFFICACY STUDY IMPLEMENTATION REVIEW PROCESS FOR DETERMINING EFFECTIVENESS OF PREMARIN FOR OSTEOPOROSIS

With the enactment of the 1962 Harris-Kefauver Amendments to the Federal Food, Drug, and Cosmetic Act, sponsors were required to demonstrate both safety and efficacy in order to market new drugs. For drugs approved between 1938 and 1962, when only the demonstration of safety was required, FDA contracted for retrospective Drug Efficacy Studies with the National Academy of Sciences/National Research Council. This action led to Drug Efficacy Study Implementation (DESI) panels to assess the efficacy of pre-1962 drugs in the marketplace. In 1972, based on a DESI review, FDA concluded that Premarin

[3] A short-acting estrogen is a drug or drug product which releases the estrogen relatively promptly after administration and which requires frequent dosing, ranging from daily to weekly.

was effective for certain indications related to menopause, and was "probably effective" for selected cases of osteoporosis. For the latter case, FDA required sponsors to submit substantial evidence of effectiveness or remove the indication from the product labeling within a certain period of time. Wyeth-Ayerst provided data to FDA in order to upgrade the osteoporosis indication; however, in 1976, the agency determined that the firm's data did not provide substantial evidence of effectiveness.

Based on published clinical study data of an estrogen product called mestranol, presented at the 1977 meeting of FDA's Endocrinologic and Metabolic Drugs Advisory Committee and the 1978 meeting of FDA's Obstetrics and Gynecology Advisory Committee, members of both committees concluded that substantial evidence was available demonstrating that estrogens, including Premarin, effectively prevented post-menopausal bone loss. Both committees also recommended a dose equivalent to 0.625 mg. Premarin for osteoporosis management as the lowest dose to assure efficacy while minimizing the risk of endometrial cancer. The FDA accepted the new osteoporosis indication for the class of non-contraceptive short-acting estrogen drugs, which included Premarin, and required that the lowest effective dose of each estrogen product in the management of osteoporosis be rigorously delineated as the basis for approval of each product.

In April 1986, based on two dose-ranging clinical studies, FDA upgraded the effectiveness status of Premarin and other drugs in its class to "effective" for use in the prevention and management of osteoporosis in post-menopausal women. One of the studies, conducted by Robert Lindsay in the late 1970's and published in 1984 (hereafter referred to as the Lindsay study), has been considered the pivotal study demonstrating the lowest effective dose of Premarin for the prevention and management of osteoporosis.

GENERIC DRUGS

The Drug Price Competition and Patent Term Restoration Act of 1984 (P.L. 98-417, dated September 24, 1984), also referred to as the Waxman-Hatch Act, amended the Federal Food, Drug, and Cosmetic Act, 21 U.S.C. 355(j)(2)A to require that generic drugs be shown to be bioequivalent to the appropriate brand name drug that has already been approved by FDA as safe and effective. By law, an applicant must demonstrate to FDA that the proposed generic drug is bioequivalent to the brand name drug, meaning that there is an absence of a significant difference in the rate and extent to which the active ingredient becomes available at the site of drug action. The applicant must also demonstrate to FDA that the proposed generic drug is a pharmaceutical equivalent of the brand name drug in terms of identity, strength, quality, and purity.

From the mid-1970's to 1991, numerous generic versions of conjugated estrogens tablets were marketed in the United States. In February 1990, however, FDA proposed withdrawing approval of generic Premarin tablets applications due to a documented lack of bioequivalence (faster rate of absorption compared to brand name Premarin) and consequent concerns about safety and efficacy. Between March and May 1991, FDA withdrew approval

for all generic applications for conjugated estrogens tablets, and these generic products were withdrawn from the market. In 1994 and 1995, respectively, two sponsors submitted to FDA applications to market generic versions of conjugated estrogens based on synthetic ingredients. However, on May 5, 1997, the Director of CDER announced that, because the active ingredients of Premarin have not been adequately defined, the agency could not at this time approve generic applications for synthetic versions of conjugated estrogens.

CITIZEN PETITION PROCESS

Through the citizen petition process, FDA allows anyone--individuals or companies--to request the agency to make changes to its regulations. The citizen petition regulations cited at 21 C.F.R. Section 10.30, require FDA to furnish a response to each petitioner within 180 days of receipt of the petition. The response will either: (1) approve the petition; (2) deny the petition; or (3) provide a tentative response.

Wyeth-Ayerst is using the citizen petition process to request FDA to recognize an ingredient of Premarin as essential. Although FDA has always recognized two active ingredients in Premarin--sodium estrone sulfate and sodium equilin sulfate--Wyeth-Ayerst submitted a citizen petition to FDA in November 1994, requesting the agency to: (1) designate a component of Premarin--DHES--as an essential (but not an active) ingredient in Premarin; and (2) not approve any generic product which does not contain DHES. The firm amended its petition in December 1996 to request FDA to recognize DHES as an active ingredient. To date, FDA has not made a decision as to whether to approve Wyeth-Ayerst's petition request.

CONGRESSIONAL CONCERNS

The House Committee on Commerce, Subcommittee on Oversight and Investigations, has raised concerns that Wyeth-Ayerst may have made misrepresentations in its submissions to FDA regarding Premarin, and that FDA may have failed to adequately review such submissions. The Subcommittee requested OIG in July 1996 to answer specific questions regarding Premarin, another Wyeth-Ayerst product called Prempro, the citizen petition related to Premarin, and generic versions of Premarin.

OBJECTIVE, SCOPE, AND METHODOLOGY

The objective of our review was to answer specific questions about FDA's regulation of Wyeth-Ayerst's conjugated estrogens product, Premarin, posed by the Chairman, House Subcommittee on Oversight and Investigations, Committee on Commerce, in a July 11, 1996 letter to the Inspector General.

To answer these questions, we reviewed applicable laws, regulations, policies, and procedures pertaining to new drug applications, supplements, and amendments. We also reviewed FDA's regulation on citizen petitions and FDA's policy on "Fraud, Untrue