SYNTHESIS OF [2,4-^{3}H] 17β-DIHYDROEQUILIN SULFATE*

Bhagu R. Bhavnani
Department of Obstetrics and Gynaecology, University of Toronto and St. Michael's Hospital, 30 Bond Street, Toronto, Ontario, CANADA, M5B 1W8.

SUMMARY

[2,4-^{3}H] 17β-dihydroequilin-3-sulfate ammonium salt suitable for in vivo pharmacokinetic studies was synthesized from [2,4-^{3}H] equilin. Sulfation of [2,4-^{3}H] equilin with pyridine-chlorosulfonic acid mixture gave in high yields [2,4-^{3}H] equilin sulfate, which was then reduced with sodium borohydride to yield [2,4-^{3}H] 17β-dihydroequilin sulfate. The reduction was sterospecific and no 17α-reduced products were formed.

Kew Words: 17β-Dihydroequilin, equilin, 17β-dihydroequilin-3-sulfate, equilin-3-sulfate, conjugated equine estrogens, ring-B unsaturated estrogens.

INTRODUCTION

Conjugated equine estrogen preparations such as Premarin (Wyeth-Ayerst, Pa., USA) contain sulfate esters of the ring-B unsaturated estrogens equilin (3-hydroxy-1,3,5-(10)7-estratetraen-17-one), equilenin (3-hydroxy-1,3,5-(10)6,8 estrapentaen-17-one), 17α-dihydroequilin(1,3,5(10)7-estratetraen 3, 17α-diol), 17α-dihydroequilenin (1,3,5(10)6,8 estrapentaen-3,17α-diol), 17β-dihydroequilin (1,3,5(10)7-estratetraen 3,17β-diol) and 17β-dihydroequilenin (1,3,5(10)6,8 estrapentaen-3, 17β-diol), and the ring-B saturated estrogens estrone, 17β-estradiol and 17α-estradiol. The major components are estrone 3-sulfate (45%), equilin 3-sulfate (25%)and 17α-dihydroequilin 3-sulfate (15%). The 17β-reduced metabolites are present in trace

***This work was supported by the Medical Research Council of Canada, Grant MT-11929.**

CCC 0362–4803/94/050439–07

Received 20 October, 1993
Revised 29 November, 1993

amounts (1). The ring-B unsaturated estrogens are excreted exclusively by the pregnant mare and they are formed by a squalene-cholesterol independent pathway (2). These drugs have been used for over 40 years for estrogen replacement therapy and for the prevention of osteoporosis and cardiovascular disease in postmenopausal women.

We have recently reported (3) that in postmenopausal women and men, approximately 30% and 2% of equilin sulfate under steady-state conditions is metabolized to 17β-dihydroequilin sulfate and 17β-dihydroequilin respectively. Bioassay data (4) indicate that 17β-dihydroequilin sulfate is over 8 times more potent uterotropic agent than equilin sulfate and estrone sulfate (4). We have also reported that among the naturally occurring estrogens 17β-dihydroequilin has the highest affinity for estrogen receptors present in the human endometrium and the rat uterus (5). Furthermore, there is some evidence that indicates orally administered 17β-dihydroequilin sulfate in doses of 0.3 to 0.4 mg/day is effective as 0.625 mg of conjugated equine estrogens for the control of menopausal symptoms (2, and references therein). There is no information available regarding the metabolic fate and pharmacokinetics of 17β-dihydroequilin sulfate in any species. To facilitate these studies, high specific activity tritiated 17β-dihydroequilin sulfate is needed. A simple method for the synthesis of high specific activity [2,4,^{3}H] 17β-dihydroequilin 3-sulfate is described.

MATERIALS AND METHODS

Purification of Steroids:

Steroids were purified by paper chromatography and by high-performance liquid chromatography (HPLC). Paper chromatography (No.3 MM paper, Whatmam, Inc., Clifton, N. J.) was performed using the following systems (vol/vol): A) isopropanol-n-butanol-ammonium hydroxide-water(45:15:1:39); B) benzene-cyclohexane-methanol-water (1:2:3:3). HPLC was performed using Beckman System Gold

Model 126, equipped with a DU 167 ultraviolet detector and a stainless steel column (4.6 mm x 250 mm) packed with ODS-C18. The HPLC solvent systems used were C) methanol -0.1M potassium phosphate (40:60); D) aectonitrile-water-acetic acid (35:65:1).

Measurement of Radioactivity:

Radioactivity was measured in a liquid scintillation spectrometer (Beckman, Toronto, Canada, Model LS 5000 TA). The radioactive material in aqueous samples was measured by counting 0.1 mL to 0.5 mL aliquots in 3.5 mL of Atomlight (Dupont - New England Nuclear - Toronto, Canada). All other samples were counted using 5 mL of toluene phosphor solution containing 0.4% Omnifluor (Dupont-NEN). All counts were corrected to 100% efficiency by external standardization.

Isotopes:

[2,4-^{3}H] Equilin (SA 43.4 Ci/mmole; Dupont-NEN) was obtained through custom synthesis and its radiochemical purity was established as described previously (6,7). Just before use, its purity was checked by HPLC using System D (retention time of equilin at a flow rate of 5 mL/min was 31 to 31.5 minutes) and was found to be over 95% pure.

Experimental

Synthesis of [2,4-^{3}H] equilin-3-sulfate ammonium salt (ammonium estra-1,3,5(10)7-tetraen-17-one-3yl sulfate).

[2,4-^{3}H] Equilin (0.5 mCi) in 1.2 mL of ethanol-benzene mixture (1:9) was diluted with 1.0 mg of unlabeled equilin to give a final specific activity of 130 mCi/mmole. The solvents were removed under vacuum and the residue was dissolved in 1.5 mL of dry pyridine and added dropwise to a previously prepared mixture of pyridine (1 mL) and chlorosulfonic acid (0.1 mL) at 4°C. The mixture was allowed to react for 15 minutes at 4°C and then warmed to 70°C until a clear solution was obtained.

After 18 hours at room temperature the pyridine was removed under vacuum and the pyridinium salt of equilin sulfate was converted to the ammonium salt by dissolving the residue in 5 mL of water and 1 mL of 7M ammonium hydroxide.

The [2,4-^{3}H] equilin sulfate ammonium salt plus unreacted [2,4-^{3}H] equilin were extracted with 1g of freshly activated Amberlite XAD-2 resin (8). After all of the steroids had been absorbed, the resin was then washed with 10% aqueous ammonium hydroxide (10 mL; 3 times), and then with 10% ammonium hydroxide in methanol (20 mL; 3 times). The methanol extract (1 x 10^9 dpm) was evaporated to dryness and the residue was partitioned between ether and water. The ether extract containing unreacted [2,4-^{3}H] equilin (6 x 10^6 dpm, approx. 0.6%) was not processed further. The aqueous extract was reextracted with XAD-2 resin and a total of 9.5 x 10^8 dpm of [2,4-^{3}H] equilin sulfate was recovered. Aliquots (1 x 10^6 dpm) were chromatographed on paper and HPLC using Systems A and C. In both systems a single peak of radioactive material (Rf in System A 0.3; retention time in System C, at a flow rate of 5 mL per minute was 12 minutes) corresponding in mobility to authentic equilin sulfate was observed. The remaining [2,4-^{3}H] equilin sulfate was used without further purification for the synthesis of [2,4-^{3}H] 17β-dihydroequilin sulfate ammonium salt as described below.

Synthesis of [2,4-^{3}H] 17β-dihydroequilin-3-sulfate ammonium salt (Ammonium estra-1,3,5(10)7-tetraen-17β-ol-3yl sulfate.

Sodium borohydride (5 mg) was added to an ice cold solution of [2,4-^{3}H] equilin sulfate ammonium salt (5.5 x 10^8 dpm) in 5 mL of methanol. The mixture was stirred for 1 hour at 4°C, following which a few drops of acetic acid were added and the methanol evaporated off under nitrogen. The residue was partitioned between ether and water so as to remove any hydrolyzed product (>1%). The [2,4-^{3}H] 17β-dihydroequilin sulfate formed was recovered from the aqueous phase by extraction with XAD-2 as described above. A total of 5.2 x 10^8 dpm was recovered

from the XAD-2 resin in the methanol extract. After removal of methanol, the residue containing [2,4-^{3}H] 17β-dihydroequilin sulfate was purified by HPLC using System C (flow rate 5.0 mL/minute). A major peak of radioactive material (retention time 8 minutes) corresponding to the mobility of 17β-dihydroequilin sulfate was observed. Fractions containing the above radioactive peak were pooled and the solvents evaporated off under vacuum. The residue was dissolved in water (10 mL) and the purified [2,4-^{3}H] 17β-dihydroequilin sulfate ammonium salt was recovered by extraction with XAD-2. A total of 3.3 x 10^6 dpm (yield 60%) of [2,4-^{3}H] 17β-dihydroequilin sulfate was obtained and its radiochemical purity established by the following criteria.

Solvolysis, Chromatography and Isotope Dilution.

A small aliquot (1 x 10^6 dpm) of [2,4-^{3}H] 17β-dihydroequilin sulfate was solvolyzed in tetrahydrofuran and perchloric acid as described previously (9). A total of 9.6 x 10^5 dpm (96%) of ether soluble product (unconjugated [2,4-^{3}H] 17β-dihydroequilin) was recovered. Aliquots of this hydrolyzed material were chromatographed on paper and HPLC using Systems B and D. A single peak of radioactive material corresponding in mobility to 17β-dihydroequilin (Rf in System B = 0.5 and retention time in system D = 18 minutes at a flow rate of 4.5 mL/minute) was observed in both systems. No 17α-dihydroequilin was detectable.

Radiochemical homogeneity of [2,4-^{3}H] 17β-dihydroequilin formed from [2,4-^{3}H] 17β-dihydroequilin sulfate was confirmed by the isotope dilution technique and was found to be over 95% pure, both before and after derivatization (Table 1).

Discussion

A number of sulfating agents have been described for the synthesis of steroid sulfate esters (10-13), some more selective than others (13). In conjugated equine estrogen preparations the estrogens are present only in the form of their 3 mono

Table 1. Proof of radiochemical purity of [2,4-^{3}H] 17β-dihydroequilin formed by solvolysis of [2,4-^{3}H] 17β-dihydroequilin sulfate.

Crystallization Number	Specific Activity (dpm/mmol x 10^5)	
	17β-Dihydroequilin[a]	17β-Dihydroequilin-3 methyl ether[b]
1	2.72	2.70
2	2.70	2.60
3	2.73	-
Calculated	2.80	2.73

a) A total of 5 x 10^4 dpm of the solvolyzed product was mixed with 50.0 mg of carrier 17β-dihydroequilin before crystallization. The calculated values are based on these figures.

b) The third crystals were methylated and the methyl ether formed was recrystallized.

sulfates (1) and as discussed above, a need arose for labeled 17β-dihydroequilin-3-sulfate. Even though we have previously synthesized high specific ^{3}H labeled 17β-dihydroequilin (5), direct sulfation was not attempted as it could result in formation of not only the monosulfates (3 or 17) but also disulfates. We have previously synthesized [2,4-^{3}H] equilin sulfate (14) and used this estrogen for the synthesis of [2,4-^{3}H] 17β-dihydroequilin-3-sulfate by sodium borohydride reduction. The [2,4-^{3}H] 17β-dihydroequilin-3-sulfate synthesized was suitable for investigation dealing with the pharmacokinetics in postmenopausal women (15), and these studies have been initiated.

REFERENCES

1) Herr, F., Revesz, C., Manson, A.J. and Jewell, J.B. Chemical and Biological Aspects of Steroid Conjugation (Bernstein, S, Solomon, S, eds.) Springer-Verlag, New York, 368 (1970).

2) Bhavnani, B.R. Endocrine Reviews. 9:396 (1988).

3) Bhavnani, B.R. and Cecutti, A. 38th Annual Meeting of the Society for Gynecologic Investigations Abstract No. 140 (1991).

4) Dorfman, R.I. and Dorfman, A.S. Endocrinology. 55:65 (1954).

5) Bhavnani, B.R. and Woolever, C.A. Steroids. 56:201 (1991).

6) Bhavnani, B.R., Sarda, I.R. and Woolever, C.A. J. Clin. Endocrinol. Metab. 52:741 (1981).

7) Bhavnani, B.R. and Woolever, C.A. Endocrinology. 108:232 (1981).

8) Bradlow, H.L. Steroids. 11:265 (1968).

9) Bhavnani, B.R. and Solomon, S. Endocrinology. 84:1230 (1969).

10) Fieser, L.F. J. Amer. Chem. Soc. 70:3232 (1948).

11) Levitz, M. Steroids. 1:117 (1963).

12) Dusza, J.P., Joseph, J.P. and Bernstein, S. Steroids. 12:49 (1968).

13) Mumma, R.D., Holberg, C.P. and Weber, W.H. Steroids. 14:67 (1969).

14) Bhavnani, B.R., Woolever, C.A., Benoit, H. and Wong, T. J. Clin. Endocrinol. Metab. 56:1048 (1983).

15) Bhavnani, B.R. and Cecutti, A. 39th Annual Meeting of the Society for Gynecologic Investigations. Abstract No. 30, March 1992.