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Determination of 17α-dihydroequilenin in rat, rabbit and monkey plasma by high-performance liquid chromatography with fluorimetric detection

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Abstract

A high-performance liquid chromatographic (HPLC) method with fluorescence detection for the determination of total (unconjugated and conjugated) 17α-dihydroequilenin in male and female rat, female rabbit and male and female rhesus monkey plasma is described here. Plasma sample preparation involved hydrolysis with enzyme (Glusulase), addition of internal standard (14β-equilenin) and solvent extraction. The extracts were chromatographed on a C_6, 5-μm reversed-phase HPLC column and detection was accomplished with a fluorescence detector operated at an excitation wavelength of 210 nm and an emission wavelength of 370 nm. The assay was linear over a range of 2.5 to 100 ng/ml in male and female rat plasma, and 5 to 500 ng/ml in female rabbit and male and female monkey plasma. The method was specific, accurate and reproducible (percent differences <14.5; coefficients of variation <9.5%) in all matrices examined. The applicability of this method was successfully tested by quantifying total plasma concentrations of 17α-dihydroequilenin in ovariectomized female rats, ovariectomized female rabbits and a normal female rhesus monkey receiving 2.0, 8.3 and 0.1 mg/kg, respectively, of 17α-dihydroequilenin sulfate intragastrically.

Keywords: 17α-Dihydroequilenin

1. Introduction

Cardiovascular diseases such as coronary artery disease and cerebrovascular disease are the leading causes of death in postmenopausal women in the United States [1,2]. High total cholesterol and low high-density lipoprotein (HDL) cholesterol to low-density lipoprotein (LDL) cholesterol ratios have long been linked with the risk of development of coronary artery disease [3,4]. Epidemiology studies indicate that estrogen replacement therapy for post-menopausal women with the conjugated equine estrogen preparation Premarin has a protective effect on coronary heart disease and recent studies clearly show that Premarin favorably alters the HDL/LDL ratios in postmenopausal women [5–9].

Premarin brand conjugated equine estrogens, which has been in use for the treatment of vasomotor symptoms and osteoporosis in post-menopausal women for more than five decades, consists of a mixture of at least ten steroids [10]. Estrone, equilin, 17α-dihydroequilin, 17α-dihydroequilenin, 17β-

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dihydroequilin, 17β-dihydroequilenin, equilenin, 17α-estradiol, 17β-estradiol and $\Delta^{8,9}$-dehydroestrone are ten steroids that are known to be present in their sulfate ester forms in Premarin. A recent study demonstrated that 17α-dihydroequilenin-3-sulfate is capable of lowering total plasma cholesterol concentrations by as much as 60–70% without inducing uterine weight gain in ovariectomized female rats [11]. In order to further understand the effects of this compound on the plasma lipid concentrations, it is important to determine its pharmacokinetic and pharmacodynamic relationships in animal models such as rats, rabbits and monkeys.

Analytical methods that have been reported in the literature are capable of quantitating only a few of the components of Premarin in biological matrices. For example, gas chromatographic methods have been developed to measure estrone and equilin in plasma of women [12,13]. Recently an HPLC method with fluorimetric detection has been reported for the analysis of estrone, equilin, 17α-dihydroequilin and 17β-estradiol in dog plasma [14]. However, no method has been available for the analysis of 17α-dihydroequilenin in biological matrices.

In this report, we describe a high-performance liquid chromatographic (HPLC) assay with fluorimetric detection for the quantitation of total (unconjugated and conjugated) 17α-dihydroequilenin in male and female rat, female rabbit and male and female monkey plasma.

2. Experimental

2.1. Chemicals and reagents

17α-Dihydroequilenin (estra-1,3,5(10),6,8-pentaene-3,17α-diol; 3,17α-dihydroxy-1,3,5(10),6,8-estrapentaene), 17α-dihydroequilenin sulfate (estra-1,3,5(10),6,8-pentaene-3,17α-diol-3-sulfate; 3,17α-dihydroxy-1,3,5(10),6,8-estrapentaene-3-sulfate) and other components of Premarin were supplied by Wyeth-Ayerst Research (Rouses Point, NY, USA). 14β-Equilenin (Isoequilenin; estra-1, 3, 5 (10) 6, 8-14β-pentaene-3ol-17-one;3-hydroxy-1,3,5(10),6,8-14β-estrapentaene-17-one, internal standard) was obtained from Steraloids (Hamilton, NH, USA). Glusulase, a *Helix pomatia* juice containing 10 000 units/ml sulfatase and 90 000 units of β-glucuronidase, was obtained from Dupont, NEN Research (Wilmington, DE, USA). Ammonium acetate was purchased from J.T. Baker (Phillipsburg, NJ, USA). All chromatographic solvents were HPLC grade from commercial sources. Heparinized plasma (control) from male and female Sprague–Dawley rats, female rabbits and male and female rhesus monkeys was obtained from Charles River Laboratories (Wilmington, MA, USA).

2.2. Instruments and chromatography

A Rainin HPXL dual pump system (Woburn, MA, USA) and a Spectra-Physics 8780 autosampler were used. A C_6, 5-μm, 15 cm×3.0 mm I.D. reversed-phase column (Column Engineering, Ontario, CA, USA) was connected to a Spectra-Physics FL 2000 fluorescence detector operated at an excitation wavelength of 210 nm and an emission wavelength of 370 nm. The data were captured using Perkin Elmer Nelson Turbochrome (Version 4.03, Nelson Analytical, Cupertino, CA, USA). The mobile phase was mixed by the HPLC pump to deliver a fluid composition of 0.05 *M* ammonium acetate, pH 3.5, acetonitrile and methanol (65:27:8, v/v/v). Mobile phase was pumped at 0.35 ml/min and all chromatography was carried out at ambient temperature.

2.3. Standard solutions

A stock solution of 100 μg/ml of 17α-dihydroequilenin and internal standard (14β-equilenin) were made in acetonitrile and stored at 4°C. Working standards were prepared by serial dilution of the stock solution in water. Calibration standards of 2.5, 5, 10, 25, 50, 75, 100, 250 and 500 ng/ml were prepared by adding 0.1 ml of each working water standard to 1 ml of control (blank) plasma. The stock solution for 17α-dihydroequilenin sulfate was made in water.

2.4. Sample preparation

Samples of 0.1 ml (rabbit) or 1 ml (rat or monkey) of plasma were added to 25 ml test tubes containing 1 ml of 0.1 *M* acetate buffer (pH 5.0) and 50 μl of Glusulase. The tubes were incubated at 37°C for 1 h.

After cooling to room temperature, diethyl ether (15 ml) was added to each tube and samples were shaken for 10 min at high speed on a mechanical shaker. After centrifugation at 3033 *g* for 10 min, the aqueous layer was frozen in a dry ice–acetone bath and the supernatant transferred to a 16×125 mm test tube. The organic phase was evaporated to dryness under nitrogen in a Zymark Turbovap (Hopkinton, MA, USA). The residues were reconstituted in 500 μl of mobile phase, filtered through a Gelman nylon acrodisc 13 syringe filter (0.45 μm pore size) and 150 μl was injected onto the column.

2.5. Specificity

The specificity of the method was evaluated by determining interference from any endogenous substances from ten individual (not pooled) rat, rabbit and monkey plasma samples. Specificity was further evaluated by determining the interference from selected Premarin components. The retention times of equilenin and 17β-dihydroequilenin were established in control plasma.

2.6. Precision and accuracy

The linearity, sensitivity, precision and accuracy of the recovery of 17α-dihydroequilenin from rat and monkey plasma were evaluated in both within-run and between-run studies. Intra- day data were provided by replicate analysis (n=3–5) of 2.5, 5, 10, 25, 50, 75, 100, 250 and 500 ng/ml concentrations of 17α-dihydroequilenin in control plasma.

Inter-day data were obtained by analysis of standards at three or more concentrations per day for 3 days in male and female monkey plasma. On each of three occasions, two or more calibration curves were analyzed and the inter-day variability was assessed from the mean of six or more determinations at each concentration.

2.7. Extraction recovery

The extraction recovery was determined by comparing the area of the 17α-dihydroequilenin peaks in extracted male rat and monkey plasma to those of directly injected analytical standard solutions. Two different concentrations (50 and 75 ng/ml) were tested in rat plasma and all concentrations in the calibration curves were examined in monkey plasma. The extraction and HPLC determinations were performed as described above.

2.8. Determination of 17α-dihydroequilenin sulfate

The utility of the method for the quantitation of 17α-dihydroequilenin sulfate in rat, rabbit and monkey plasma was evaluated. Plasma samples containing three different concentrations (25, 50 and 75 ng/ml or 45, 67.5 and 90 ng/ml) of 17α-dihydroequilenin sulfate were hydrolyzed with Glusulase as described above. Intra-day precision and accuracy data were obtained by analyzing four or five samples of each concentration. Inter-day precision and accuracy data were obtained by analysis of samples (n=6 or 7) prepared on three separate occasions.

2.9. Application

The application of the method for pharmacokinetic studies was tested by analyzing plasma samples from rats, rabbits and a monkey receiving 17α-dihydroequilenin sulfate. Female ovariectomized rats, female ovariectomized rabbits and a normal female monkey were dosed orally with 2.0, 8.3 and 0.1 mg/kg, respectively, of 17α-dihydroequilenin sulfate and blood was collected at 0 h (pre-dose), 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 12.0, 15.0 and 24.0 h post-dose. Blood was centrifuged at 3033 *g* for 15 min and plasma was separated and kept frozen at −20°C until assayed for total 17α-dihydroequilenin concentrations.

2.10. Calculations

Calculations were performed by weighted $(1/y^2)$ least square linear regression analysis of the peak height ratios of 17α-dihydroequilenin/14β-equilenin against the concentrations of 17α-dihydroequilenin. [15]

3. Results and discussion

3.1. Chromatography

The chemical structures of 17α-dihydroequilenin, 17α-dihydroequilenin-3-sulfate and the internal standard 14β-equilenin are shown in Fig. 1. Fluorescence detection provided greater sensitivity for 17α-dihydroequilenin than any other mode. 17α-Dihydroequilenin and the internal standard were well separated by the column used in this method with retention times of approximately 19.5 min and 24 min for the analyte and the internal standard, respectively. Fig. 2 shows chromatograms obtained from control (blank) plasma containing internal standard, control plasma containing analyte and internal standard, and a plasma sample obtained 2 h after administration of 17α-dihydroequilenin sulfate at 2.0 mg/kg intragastrically to an ovariectomized female rat.

3.2. Linearity and specificity

The calibration curves were linear over the concentration ranges of 2.5 to 100 ng/ml in rat plasma and 5 to 500 ng/ml in rabbit and monkey plasma with a mean correlation coefficient (r^2) of >0.985

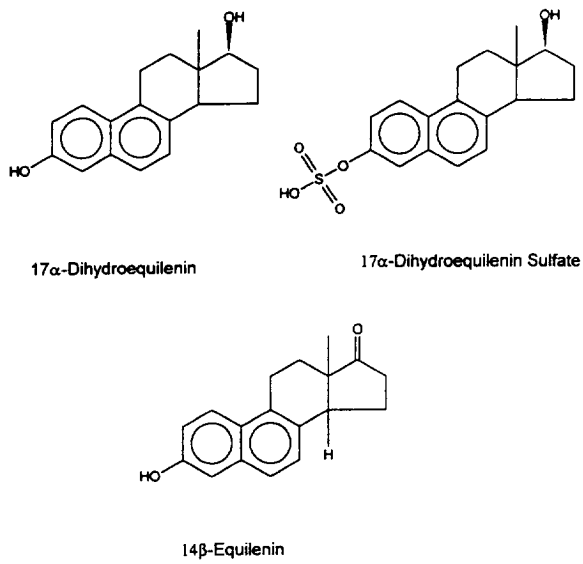

Fig. 1. Chemical structure of 17α-dihydroequilenin, 17α-dihydroequilenin-3-sulfate, and 14β-equilenin, internal standard.

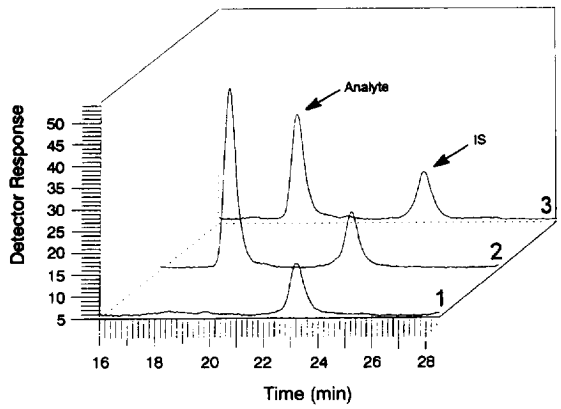

Fig. 2. Chromatograms obtained from a control female rat plasma containing internal standard (1), control female rat plasma containing 50 ng/ml calibration standard (2), and plasma from a female ovariectomized rat taken 4 h after a 2.0 mg/kg intragastric dose of 17α-dihydroequilenin sulfate.

(Table 1). The specificity of the method was demonstrated by the absence of endogenous peaks with retention times corresponding to 17α-dihydroequilenin or internal standard in control plasma. Other Premarin components, specifically, equilenin and 17β-dihydroequilenin, also did not interfere with the detection of 17α-dihydroequilenin.

3.3. Precision and accuracy

The intra-day precision of the method was assessed by calculating the coefficient of variation for replicate samples (n=3–5) at concentrations of 2.5, 5, 10, 25, 50, 75 and 100 ng/ml in male and female rat and at 5, 10, 25, 50, 75, 100, 250 and 500 ng/ml in female rabbit, male and female monkey plasma. The coefficient of variation ranged from 0.1 to 9.5% over the entire linear range in the matrices tested and was independent of 17α-dihydroequilenin concentration. The accuracy of the method, defined as the percent difference between the mean 17α-dihydroequilenin found and the theoretical, ranged from −4.9 to 14.7% and was not concentration dependent.

The inter-day precision and accuracy data were derived from the analysis of replicates (n=6–8) at 5, 10, 25, 50, 75, 100, 250 and 500 ng/ml concentrations of 17α-dihydroequilenin in male and female rhesus monkey plasma on three separate days with

Table 1
Intra-day calibration curve parameters for 17α-dihydroequilenin (n=3–5)

Matrix	Slope (mean ±S.D.)	C.V. (%)	Mean intercept	Mean correlation coefficient (r^2)
Male rat plasma	0.994±0.009	0.91	0.062	0.998
Female rat plasma	0.931±0.075	8.1	0.591	0.985
Female rabbit plasma	0.986±0.135	13.7	0.715	0.995
Male monkey plasma	0.949±0.038	4.0	1.002	0.991
Female monkey plasma	0.936±0.017	1.8	1.342	0.991

two or more determination on each day. Precision varied between 1.7% to 8.7% and accuracy from −4.6 to 4.3%. The percent difference was not concentration dependent.

3.4. Extraction recovery and stability

The recovery of 17α-dihydroequilenin was determined by comparing the peak area of the compound extracted from plasma to that of pure reference standard working solutions injected directly on the HPLC column. The mean recovery of 17α-dihydroequilenin from rat plasma was about 74% and from monkey plasma was between 60 to 67%. The analyte stability was tested under different conditions. Plasma extracts and standards were kept at 4°C and room temperature. HPLC analysis showed that there was no decomposition of the drug in extracts or standard solutions at 4°C over a 24-h period.

3.5. Determination of 17α-dihydroequilenin sulfate

Estrogens are known to circulate in blood both in free phenolic forms (unconjugated) and conjugated forms (sulfates and glucuronides). However, the majority of the compounds exist as the sulfate conjugates in blood [16]. Therefore, the aim of the study was to develop and validate an assay for total dihydroequilenin which would include both the unconjugated and conjugated compounds. To accomplish this, a method was employed utilizing enzyme hydrolysis for quantitating 17α-dihydroequilenin sulfate.

Intra-day precision and accuracy were determined by analyzing replicate samples (n=4–5) at three different concentrations in male and female rat plasma, female rabbit plasma and male and female monkey plasma. The coefficients of variation and percent difference did not exceed 7% and 9%, respectively, in any of the matrices examined (Table 2). Inter-day precision and accuracy of 17α-dihydroequilenin sulfate were determined by analyzing two or more replicates per day at three different concentrations in female rat, female rabbit and male and female monkey plasma. The results are shown in Table 3. The coefficients of variation and percent difference did not exceed 8% and 9%, respectively.

Although the time course of hydrolysis of 17α-dihydroequilenin sulfate was not determined in this study, the optimum conditions for the hydrolysis of the estrogen sulfates estrone sulfate and equilin sulfate have previously been established. The volume of enzyme (50 μl of Glusulase) and the duration of incubation (1 h) that were used for the hydrolysis of 17α-dihydroequilenin were found to be optimum for the hydrolysis of these two estrogen sulfates up to a concentration of 1 μg/ml. Therefore, the hydrolysis of 17α-dihydroequilenin sulfate is expected to be complete under the conditions used in this method.

3.6. Application of the method for pharmacokinetic studies

The method has been used successfully to quantify total plasma concentrations of 17α-dihydroequilenin in rats, rabbits and monkey following 0.1–8.3 mg/kg, intragastric doses. Fig. 3 shows plasma concentrations of total 17α-dihydroequilenin in ovariectomized female rats, ovariectomized female rabbits and a normal female monkey, receiving 2.0, 8.3 and 0.1 mg/kg, ig of the 17α-dihydroequilenin sulfate, respectively. Maximum (C_{max}) concentrations were reached between 2 and 6 h (t_{max}) after dosing. In

Table 2
Intra-day precision and accuracy of the HPLC assay for 17α-dihydroequilenin sulfate (n=4–5)

Matrix	Added (ng/ml)	Concentration found (mean±S.D.) (ng/ml)	Precision (% C.V.)	Accuracy (% difference)
Male rat plasma	25	25.2±0.6	2.0	0.9
	50	49.2±1.8	3.6	−1.6
	75	70.1±2.1	3.1	−6.5
Female rat plasma	25	23.1±1.1	4.8	−7.7
	50	49.5±0.9	1.9	−0.8
	75	76.5±5.2	6.8	2.0
Female rabbit plasma	45	48.5±2.2	4.6	7.8
	67.5	73.0±1.8	2.5	8.2
	90	94.3±1.6	1.2	4.8
Male monkey plasma	45	47.2±2.2	4.7	4.8
	67.5	61.4±3.3	5.4	−9.0
	90	82.6±4.4	5.3	−8.2
Female monkey plasma	45	45.8±2.2	4.9	1.8
	67.5	66.6±1.3	1.9	−1.3
	90	84.3±1.7	2.0	−6.3

rats, rapid absorption followed by a rapid decline and a secondary peak were seen. Total drug concentrations were detectable up to 15 h after dosing in rats and up to 24 h in rabbits and the monkey. The concentrations in all samples, except the 15-h sample from rats, were well within the range of the standard curves, indicating that this method is suitable for pharmacokinetic evaluations in all three species.

Table 3
Inter-day precision and accuracy of the HPLC assay for 17α-dihydroequilenin sulfate (n=6–7). Results of validation study over a three-day period

Matrix	Added (ng/ml)	Concentration found (mean±S.D.) (ng/ml)	Precision (% C.V.)	Accuracy (% difference)
Female rat plasma	25	23.3±1.2	5.0	−6.6
	50	49.4±0.8	1.7	−1.2
	75	74.5±5.5	7.4	−0.6
Female rabbit plasma	45	47.7±2.1	4.5	6.0
	67.5	71.0±3.4	4.8	5.2
	90[a]	92.5±4.2	4.9	2.8
Male monkey plasma	45	47.2±2.2	4.7	4.8
	67.5	61.4±3.3	5.4	−9.0
	90	82.6±4.4	5.3	−8.2
Female monkey plasma	45	45.8±2.2	4.9	1.8
	67.5	66.6±1.3	1.9	−1.3
	90	84.3±1.7	2.0	−6.3

[a] n=5

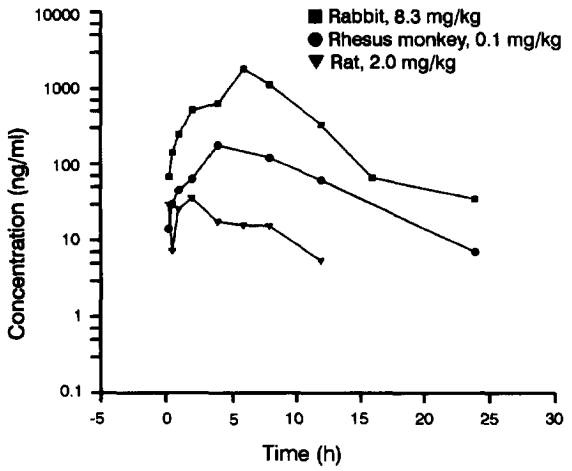

Fig. 3. Plasma concentrations of total 17α-dihydroequilenin in female ovariectomized rats, female ovariectomized rabbits and a normal female rhesus monkey receiving 2.0, 8.3 or 0.1 mg/kg of 17α-dihydroequilenin sulfate intragastrically.

4. Conclusion

A high-performance liquid chromatography method has been developed and validated for the quantitation of total 17α-dihydroequilenin in rat, rabbit and monkey plasma. Extraction of control plasma demonstrated no interference from endogenous substances or other estrogenic components in Premarin. The method met both precision and accuracy requirements. As a result, reliable quantitation of 17α-dihydroequilenin can be achieved over the range of 2.5 to 100 ng/ml in rat plasma and 5 to 500 ng/ml in rabbit and monkey plasma. The method has been successfully utilized in determining 17α-dihydroequilenin in female ovariectomized rats, female ovariectomized rabbits and normal female monkey plasma from pharmacokinetic studies.

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