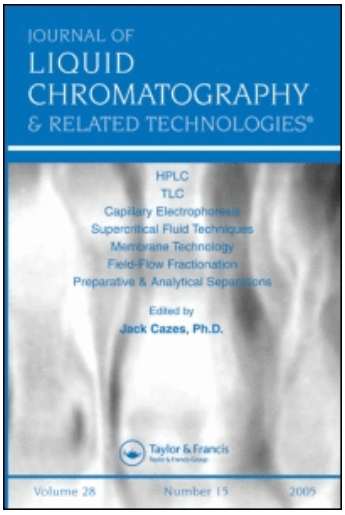

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Separatory Determination of Biliary Metabolites of Equilin in Rat by High-Performance Liquid Chromatography

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SEPARATORY DETERMINATION OF BILIARY METABOLITES OF EQUILIN IN RAT BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A method for the separation and determination of biliary metabolites of equilin in rat by high-performance liquid chromatography with electrochemical detection has been developed. After extraction of equine estrogens in bile with a C_{18} bonded silica cartridge, conjugated metabolites were hydrolyzed with sulfatase containing β-glucuronidase and free estrogens liberated were then separated on a reversed phase C_{18} column, the limit of detection being in the range of 5-20 pg. The transformation of equilin administered orally into 2-methoxy derivatives of equilin, equilenin and their 17β-reduced metabolites is also discussed.

INTRODUCTION

Equine estrogens are ring B unsaturated steroids formed through a squalene-cholesterol independent pathway and are

excreted exclusively into urine of pregnant mares as their sulfates (1). These estrogens have widely been used for treatment of postmenopausal and estrogen deficient women (2,3). Recently, a strong association between equine estrogen replacement therapy and endometrial cancer has been demonstrated (4-7). To assess the biological and endocrine potencies, clarifying the biotransformation of these estrogens is needed. However, metabolism of these equine estrogens still remains unclear, though that of classical estrogens such as estrone and estradiol in man and rodent has been well established (8). Bhavnani et. al have shown that after administration of [^{3}H] equilin and its sulfate into postmenopausal women and men, polar metabolites were excreted in urine (9,10). But the precise knowledges on the transformation of equine estrogens during circulation in the body has been spaced. It is generally accepted that both endogenous and exogenous estrogens are excreted to a considerable extent into bile and are partly reabsorbed from the intestine. Accordingly, a reliable method for the qualitative and quantitative analysis of metabolites in bile is needed for clarifying the metabolic fate of equine estrogens.

In recent years, various chromatographic methods, gas-liquid chromatography (GC), GC-mass spectrometry and high-performance liquid chromatography (HPLC), have been used for the simultaneous determination of steroid hormones in biological fluids. Among these methods, HPLC with electro-

chemical detection (ECD) is believed to be a powerful tool for the separation and determination of phenolic steroids with reliable sensitivity and specificity. The present paper deals with the high-performance liquid chromatographic determination of biliary metabolites of equilin in rat.

EXPERIMENTAL

Instruments

The apparatus used for this work was a Waters Model 510 solvent delivery system (Millipore-Waters, Milford, MA, U.S.A.) equipped with a Coulochem Model 5100A electrochemical detector (Environmental Sciences Assoc. Inc., Bradford, MA, U.S.A.). The potential voltage of the detector for 1st electrode for screening was set at 0.1 V *vs.* Ag/AgCl electrode. A Cosmosil 5C18-AR (5μm) column (15 cm x 4.6 mm i.d.) (Nacarai Tesque Inc., Kyoto, Japan) was used at ambient temperature.

Materials

Equilin (Eq) and equilenin (Equ) were kindly donated by Ayerst Laboratories (New York, NY, U.S.A.). Estrone (E_1) and estradiol (E_2) were purchased from Teikoku Hormone Mfg. Co. (Tokyo, Japan). 17β-Dihydroequilin (17β-DHEq) and 17β-dihydroequilenin (17β-DHEqu) were obtained by sodium borohydride reduction of Eq and Equ, respectively. 1-Methylestrone

was prepared in these laboratories. 2-Methoxyestrone (2-$MeOE_1$), 2-methoxyestradiol (2-$MeOE_2$), 2-methoxyequilin (2-MeOEq), 2-methoxyequilenin (2-MeOEqu) and their 17β-hydroxyl derivatives (2-MeODHEq and 2-MeODHEqu) were synthesized as reported previously (11). The reagents used were of analytical reagent grade. Solvents were purified by distillation and degassed by vacuum evacuation prior to use. The sulfatase (EC 3.1.6.1) preparation derived from Helix pomatia (Type H-5) was supplied by Sigma (St. Louis, MO, U.S.A). Piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20, 0.6 meq./g) was prepared as reported (12). A Sep-Pak C_{18} cartridge (Millipore-Waters) was washed successively with methylene chloride (5 ml), ethanol (10 ml), H_2O (15 ml), 5% bovine serum albumin (3 ml), H_2O (15 ml), ethanol (10 ml) and then H_2O (15 ml) prior to use. All glasswares used were silanized with trimethylchlorosilane.

Collection of rat bile

Male Wistar rats weighing ca. 250 g were anesthetized with diethyl ether, cannulated to the bile duct with polyethylene tube by surgical operation and housed in Bollman cages for collection of bile. All animals were starved overnight prior to administration of equilin. A suspension of equilin (10 mg) per head in dimethylsulfoxide (50 μl)-saline (350 μl)-Tween 80 (100 μl) was given orally to rat, and bile was collected in a test

tube containing 500 μl of 30 mM ascorbic acid in 0.15 M acetate buffer (pH 5.0) over a period of 24 h following administration of equilin.

Determination of metabolites in bile

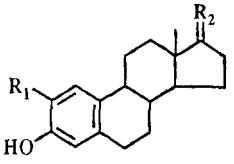
To a 10 μl aliquot of rat bile was added 1-methylestrone (100 ng) and following dilution with 0.15 M acetate buffer (pH 3.0, 2 ml) containing 2% ascorbic acid, the mixture was passed through a Sep-Pak C_{18} cartridge. After washing with distilled water (10 ml), estrogens were eluted with methanol (5 ml) and the eluate was evaporated *in vacuo*. The residue was dissolved in 0.15 M acetate buffer containing 2% ascorbic acid (pH 5.0, 2 ml) and incubated with sulfatase (500 Fishman units) at 37 °C for 15 h. The whole was applied to a Sep-Pak C_{18} cartridge in the manner described above, for extraction of liberated steroids. The hydrolyzate in 90% ethanol (1 ml) was applied to a column (18 mm x 6 mm i.d.) of PHP-LH-20 (100 mg). Estrogens were eluted with 90 % ethanol (5 ml) and the eluate was evaporated *in vacuo*. The residue was redissolved in acetonitrile (1 ml) and then subjected to the HPLC analysis.

Recovery test

A synthetic mixture of each estrogen (100 ng) dissolved in 10 μl of bile and the whole was then subjected to the proposed method. Recoveries were calculated against a standard mixture of estrogens carried through the procedure.

RESULTS AND DISCUSSION

Our initial effort was directed toward the development of efficient chromatographic conditions for the determination of E_1, Eq, Equ, related 2-methoxy derivatives and their 17β-hydroxylated compounds listed in Fig. 1 with a reversed phase column. The separation of estrogens was influenced by species of salts in a mobile phase. The addition of disodium hydrogen or ammonium dihydrogen phosphate in a mobile phase resulted in the insufficient resolution of estrone, equilin and/or equilenin. On the other hand, these estrogens were satisfactorily resolved with a mobile phase including sodium acetate. Therefore, the effect of pH of a mobile phase on k' values of estrogens were examined on a Cosmosil 5C18-AR column with 0.5% sodium acetate-acetonitrile as a mobile phase. The k' values obtained were plotted against pH of the buffer in a mobile phase. As shown in Fig. 2, increasing k' values with increasing pH in the range of 3.0-6.0 were observed for all the steroid examined. The almost identical elution order was found for guaiacol estrogens (2-MeOEqu>2-MeOEq>2-MeOE_1 and 2-MeODHEqu>2-MeODHEq>2-MeOE_2) and related phenolic compounds (Equ>Eq>E_1 and DHEqu>DHEq>E_1) according to the structure of ring B and the substituent at C-17. This may be due to hydrophobicity of a double bond in the B ring for Eq and a naphthalene ring for Equ. Although almost all compounds were

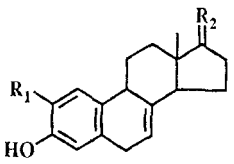


E_1 : R_1=H, R_2=O

E_2 : R_1=H, R_2=β-OH

2-MeOE_1 : R_1=OCH_3, R_2=O

2-MeOE_2 : R_1=OCH_3, R_2=β-OH

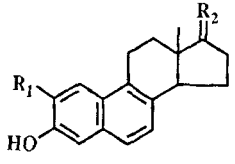


Eq : R_1=H, R_2=O

17β-DHEq : R_1=H, R_2=β-OH

2-MeOEq : R_1=OCH_3, R_2=O

2-MeODHEq : R_1=OCH_3, R_2=β-OH



Equ : R_1=H, R_2=O

17β-DHEqu : R_1=H, R_2=β-OH

2-MeOEqu : R_1=OCH_3, R_2=O

2-MeODHEqu : R_1=OCH_3, R_2=β-OH

Fig. 1. Structures of equine estrogens and related compounds.

well resolved by the use of a binary solvent system with 0.5% sodium acetate-acetonitrile or -methanol, insufficient resolution of DHEq and 2-MeODHEqu was attained. Accordingly, a ternary solvent system consisting of 0.5% sodium acetate, acetonitrile and tetrahydrofuran (THF) or methanol were investigated. The effect of the content of THF or methanol in acetonitrile on a k' value relative to that of 1-methylestrone

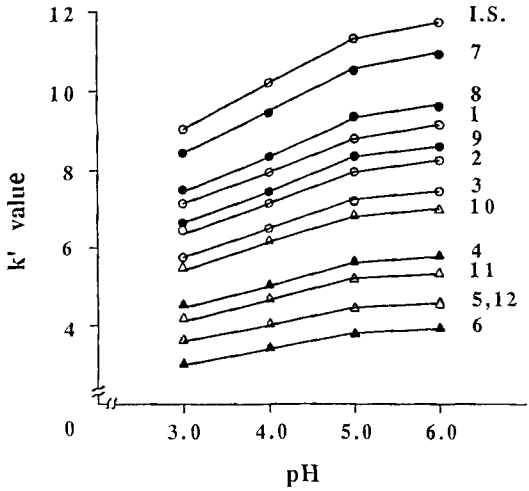


Fig. 2. Effect of pH of mobile phase on k' values of estrogens. Conditions: column, Cosmosil 5C18-AR; mobile phase, 0.5% sodium acetate-acetonitrile (60:40, v/v), flow rate, 1.0 ml/min. 1=E_1, 2=Eq, 3=Equ, 4=E_2, 5= 17β-DHEq, 6= 17β-DHEqu, 7=2-MeOE_1, 8=2-MeOEq, 9=2-MeOEqu, 10=2-MeOE_2, 11=2-MeODHEqu, 12= 2-MeODHEqu, IS=1-methylestrone.

was examined with the constant ratio of an organic solvent and a buffer solution (40:60, v/v). As depicted in Fig. 3, the remarkable variance was found for THF with respect to the feeble effect for methanol. The relative k' values of E_2, DHEq, DHEqu and corresponding 17-oxo compounds increased remarkably with a increasing the ratio of THF to acetonitrile. On the contrary, the decreasing values for 2-methoxy derivatives having a 17-oxo group were found, while those with a 17β-hydroxyl group showed almost constant k' values.

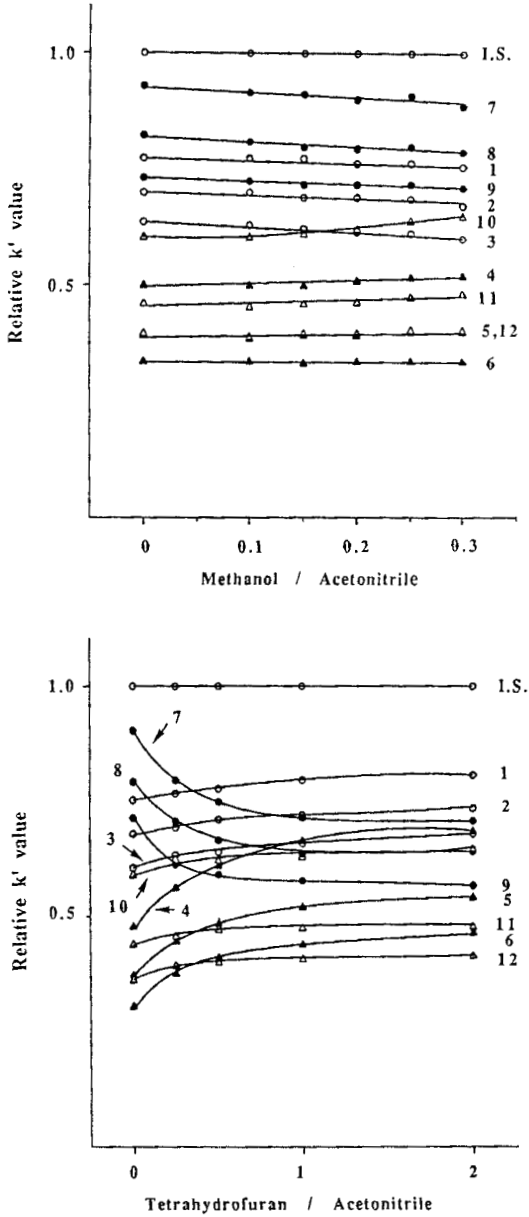


Fig. 3 Effect of content of methanol (A) or tetrahydrofuran (B) in CH_3CN on relative k' values of estrogens. Abbreviations and conditions the same as in Fig 2.

These chromatographic behaviors would be helpful for structural elucidation of equine estrogens in biological fluids. The suitable ratio of THF to acetonitrile in a mobile phase for the separation of DHEq and 2-MeODHEq was found to be in the range of 0.25-1.0. The combined use of binary and ternary solvent systems may serve for the qualitative and quantitative analysis of these estrogens with high reliability. A typical chromatogram obtained with authentic specimens is shown in Fig. 4.

The next effort was directed to investigate the electrochemical properties of equine estrogens. The effect of the applied potential on the sensitivity was tested in the range of 0.3 - 0.8 V *vs.* the silver-silver chloride electrode. The hydrodynamic voltammograms obtained with 1 ng each of these compounds per injection are shown in Fig. 5. It was found that responses of guaiacol estrogens increased linearly up to 0.5 V and then reached a plateau, and, on the other hand, phenolic compounds showed the increasing responses up to 0.8 V(13). From these results, the applied potential was set at 0.7 V. A calibration graph was constructed by plotting the peak area of each compound to that of the internal standard, a linear response to each compound being observed up to 1 ng. The detection limits were estimated to be 5 pg for Equ, DHEq and 2-MeOEqu, and to be lower than 20 pg for other steroids (signal to noise ratio = 5 at 10 nA full-scale).

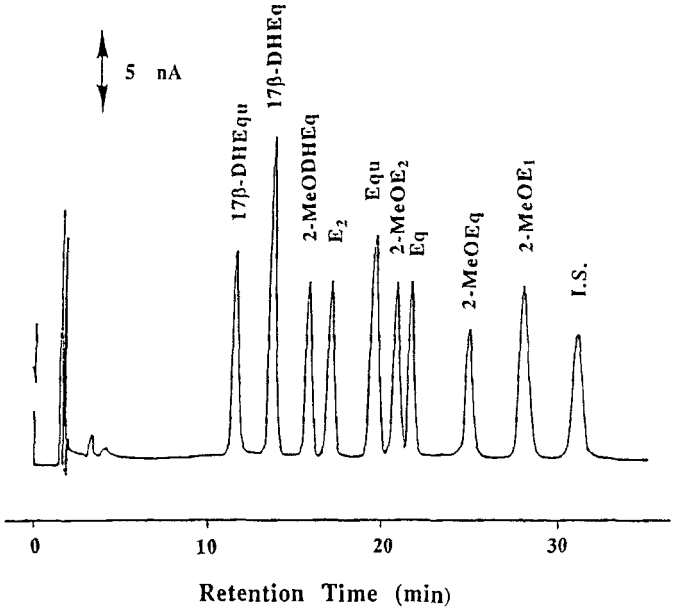


Fig. 4 Typical high-performance liquid chromatogram of the mixture of standard estrogens.
Conditions: column, Cosmosil 5C18-AR; mobile phase, 0.5% acetate buffer (pH 5.0)-methanol-acetonitrile (60:9:30, v/v/v); flow rate, 1.0 ml/min.

The present method was then applied to the characterization and determination of biliary metabolites in rat following oral administration of Eq. The separation and determination of trace compounds in biological fluids is markedly influenced by the clean-up procedure employed. For this purpose, an octadesylsilyl bonded silica cartridge was used for extraction and concentration of estrogens in bile prior to the HPLC analysis. A hundredth milliliter of a bile specimen was subjected to the

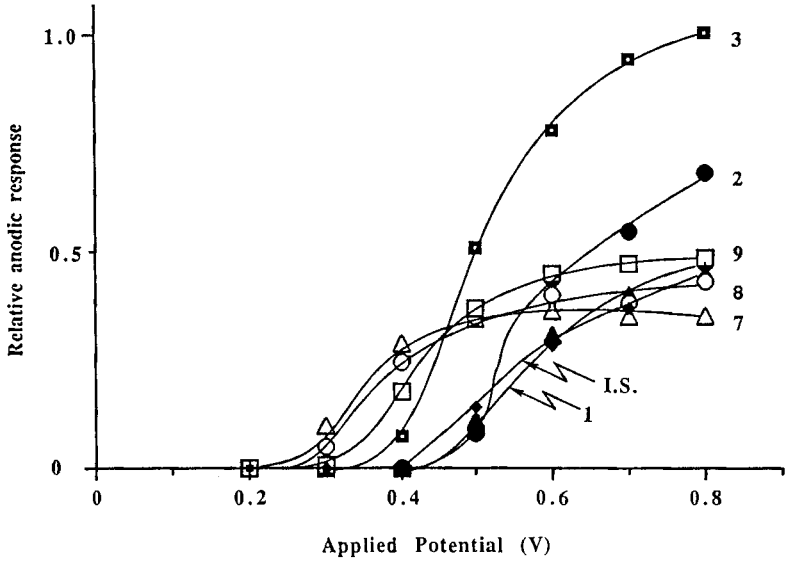


Fig. 5. Hydrodynamic voltamograms of typical estrogens. The maximum response of each steroid is arbitrarily taken as 1.0.
Abbreviations and conditions the same as in Fig. 2 and 4, respectively.

clean-up procedure with a Sep-Pak C_{18} cartridge and the enzymatic hydrolysis with sulfatase containing β-glucuronidase. The liberated steroids were further purified by ion exchange chromatography on a lipophylic gel, PHP-LH-20 to remove co-existing substances and then subjected to the HPLC separation with ECD. The recovery rates carried through the procedure described above were estimated by determining the amount of representative compounds added to a bile specimen. As listed in Table 1, each compound spiked in bile was recovered at a rate of more than 89%.

TABLE 1 Recovery of Equine Estrogens Added to Rat Bile

Compound	Equine estrogen ($ng \cdot ml^{-1}$)		Recovery ± S.D.[a]
	Added	Found	(%)
Eq	50.0	44.6	89.2 ± 4.6
17β-DHEq	50.0	44.5	88.9 ± 3.9
2-MeOEq	50.0	44.3	88.5 ± 5.1
2-MeODHEq	50.0	46.5	93.0 ± 5.7
Equ	50.0	49.7	99.4 ± 3.5
17β-DHEqu	50.0	49.0	98.0 ± 5.6
2-MeOEqu	50.0	49.7	99.4 ± 1.8
2-MeODHEqu	50.0	47.1	94.2 ± 2.6

[a] n=6

A chromatogram of equine estrogens in rat bile is illustrated in Fig. 6. Equ, as a main component, 2-MeOEqu and their 17β-reduced compounds are observed with Eq. The result with the observation of ring B dehydrogenated metabolites is the same with previous findings demonstrated with human endometrium (14) and rat liver (15), and strongly indicates the presence of enzyme, 6,8(9) steroid dehydrogenase. In accordance with the previous study with the pregnant mare (16), formations of B ring saturated estrogens, namely E_1 and E_2, were not found. A conspicuous feature is the new finding that equilin can be metabolized to 2-MeOEq and 2-MeOEqu, which are further

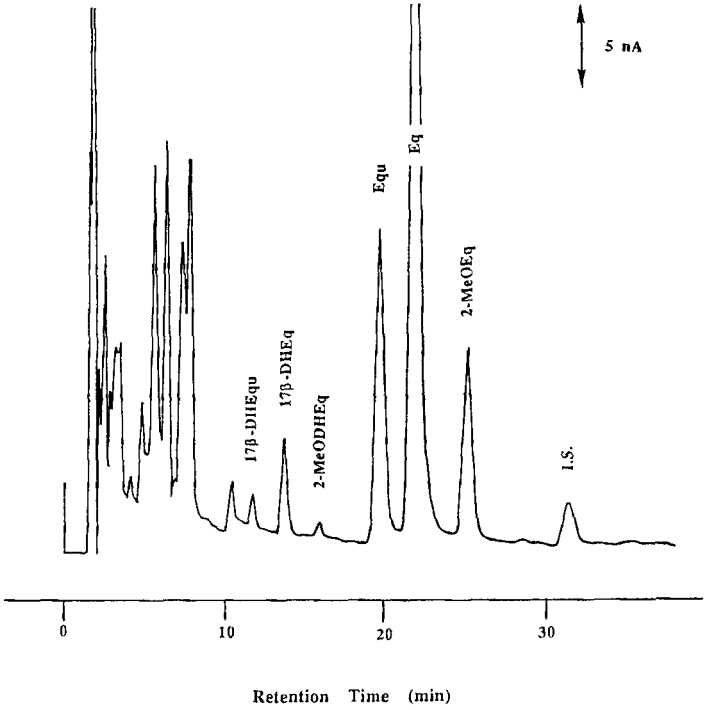


Fig. 6. High-performance liquid chromatogram of estrogens in rat bile after administration of equilin. Conditions the same as in Fig. 4.

metabolized to 17β–reduced compounds in a certain degree. This is the first demonstration of *in vivo* formation of 2-methoxylated metabolites from equilin administered, though the *in vitro* 2- and 4-hydroxylation of equine estrogens have been previously indicated in baboon liver (17). At present, it remains unknown whether 2-MeOEqu is formed from 2-MeOEq or Equ. It is well documented that conversion of phenolic

steroids to catecholic estrogens is major metabolic pathway in man and rodents (8). From these points of view, the present results strongly suggest that the catecholic Eq and/or Equ are formed as intermediary products during circulation in the body. The metabolism of catechol equine estrogens involving O-methylation appears to be an attractive to be resolved.

Recently, it has been shown that administration of [^{3}H]Eq to postmenopausal women and men resulted in the formation of DHEq, DHEqu, and Equ, which were isolated from plasma and urine (10,18). The present investigation indicates that all three metabolites can be formed in rat. It is still unknown that the enzyme involved in interconversion of equilin and equilenin into their 17β-reduced estrogens is identical with 17β-hydroxysteroid dehydrogenase.

Further studies are being conducted to clarify metabolic disposition of equine estrogens.

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