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Determination using liquid-chromatography–electrospray tandem mass spectroscopy of ethinylestradiol serum pharmacokinetics in adult Sprague–Dawley rats

Nathan C. Twaddle[a], Mona I. Churchwell[a], Retha R. Newbold[b], K. Barry Delclos[a], Daniel R. Doerge[a],*

[a]*Division of Biochemical Toxicology, National Center for Toxicological Research, Jefferson, AR 72079, USA*
[b]*Developmental Endocrinology Section, Laboratory of Molecular Toxicology, Environmental Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, USA*

Abstract

The pharmacokinetics of ethinylestradiol (EE2), a potent synthetic estrogen, was investigated in male and female Sprague–Dawley rats as part of a series of endocrine-active compounds, including genistein and nonylphenol. A method based on solid-phase extraction and LC with negative ion electrospray tandem mass spectrometric detection was developed and validated. The limit of detection in untreated rat serum was below 0.01 ng/ml (0.03 nM), the limit of quantification was 0.03 ng/ml (0.10 nM), the intra- and inter-day precision was 2–9%, and the intra- and inter-day accuracy was 89–94%. This method was used to determine the serum pharmacokinetics of EE2 in rats following oral gavage administration of 1 mg/kg body weight. EE2 was present in serum primarily in the unconjugated form at concentrations below 0.5 ng/ml. The maximal serum concentration was proportional to dose over the range of 0.04–0.5 mg/kg body weight and pharmacokinetic parameters were determined using model-independent analysis. Significant sex differences were observed for elimination half-times and volumes of distribution, but not for total serum clearance or maximal concentrations. The pharmacokinetic analysis of EE2 will be useful for comparing the toxicological effects of EE2 to those of other environmental estrogens in related rodent endocrine disruptor studies.
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1. Introduction

Ethinylestradiol (EE2) is a potent synthetic estrogen that is widely used therapeutically, mainly in oral contraceptives. Primarily because of its high estrogenic potency, EE2 was included as a test compound in a series of studies designed to evaluate the developmental, reproductive, and chronic toxicities of a series of hormonally active compounds with estrogenic activity. The other test chemicals, which included the soy isoflavone genistein and the detergent decomposition product nonylphenol, are less potent as estrogens and differ in their relative binding affinities for the α and β estrogen receptors

[1–3]. In addition to serving as a positive control estrogen for these animal studies, there was also concern that the developing human fetus may be exposed to EE2 as a result of maternal ingestion of oral contraceptives during pregnancy. The literature is mixed on whether in utero exposure to oral contraceptives containing EE2 causes malformations: some studies suggest that no malformations occur [4,5;] others have reported an association (see Ref. [5]); and a recent study in mice has indicated reproductive tract alterations occurred in males despite the absence of gross malformations following sub-clinical doses of EE2 [6].

The considerations used in selecting the EE2 doses for the present study were to utilize high enough doses that, based on the results of a dose range finding study in adult rats (Delclos, unpublished data), did not produce pronounced toxicity in the parental generation but would produce effects in progeny. The lower doses were selected to approximate human therapeutic exposures, which are less than 1 μg/kg body weight daily for oral contraceptives. The goals for assays of this series of estrogenic chemicals included comparison of the spectrum and dose dependence of effects produced in the test animals as well as identifying potential hazards of exposure to these compounds in humans. Such comparisons require detailed knowledge of the pharmacokinetics and metabolic disposition of the test compounds. Previously we reported pharmacokinetic analyses for genistein [7] and nonylphenol [8]. Despite the widespread use of EE2, there is relatively little information in the open literature on the pharmacokinetics and disposition of EE2 in rodents. This paper reports a new sensitive and specific method for the quantification of EE2 in rat serum using isotope dilution LC–ES/MS/MS and its application to pharmacokinetic analysis in male and female rats.

2. Experimental

2.1. Reagents

Ethinylestradiol (17α-ethynyl-1,3,5(10)-estriene-3,17β-diol) and all biochemical reagents were purchased from Sigma (St. Louis, MO, USA). Labeled

EE2 (20, 21-^{13}C$_2$, 99%) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). All solvents were HPLC-grade and Milli-Q water was used throughout.

2.2. Liquid chromatography

A Waters Alliance 2790 separation module (Waters, Milford, MA, USA) was used at a flow-rate of 0.3 ml/min with a mobile phase step gradient starting at 55% acetonitrile in water (v/v) for 3.6 min followed by 100% acetonitrile for 3 min followed by reequilibration for 5.3 min on an Xterra RP-18 (2.1× 150 mm, 3.5 m particle size; Waters) using a column temperature of 40 °C.

2.3. Mass spectrometry

A Micromass Quattro Ultima (Micromass, Beverly, MA, USA) triple quadrupole mass spectrometer equipped with an electrospray probe was used in multiple reaction monitoring (MRM) mode for analysis of negative ions. The optimal MRM transitions (i.e. those giving the maximal responses) for EE2 and ^{13}C$_2$-EE2 were determined to be m/z 295→145 and 297→145, respectively (i.e. [M–H]–→7,8-dihydronaphth-2-ol), using dwell times of 0.25 s, argon collision gas at 2.7×10^{-3} bar, nitrogen as both cone gas (25 l/h) and desolvation gas (742 l/h), and source and desolvation temperatures of 120 and 450 °C, respectively. A sampling cone-skimmer potential of 80 V and a collision energy of 46 eV were used throughout. Resolution was set to give peak widths at half-height of 0.9 Th for product and precursor ions.

2.4. Characterization of labeled internal standard

The chemical concentration of ^{13}C$_2$-EE2 was determined by comparing LC–UV (280 nm) responses with a solution of authentic unlabeled EE2 prepared by accurate weighing. No unlabeled EE2 was observed in the ^{13}C$_2$-EE2 (<0.1%). The natural abundance isotopic contribution of unlabeled EE2 (M+2=298 dalton) to the labeled responses was determined using full scan ES/MS and the 2.6% contribution from unlabeled responses was subtracted from the integrated areas of all ^{13}C$_2$-EE2

traces. A plot of corrected response ratios for labeled vs. unlabeled EE2 was linear over the concentration range of 1–500 pg unlabeled plus 100 pg $^{13}C_2$ with a slope of 1.08 and a correlation coefficient of 0.997.

2.5. Solid phase extraction

Thawed serum samples (75 µl), to which 100 pg labeled EE2 internal standard was added, were diluted in citrate buffer (1 ml total, 25 mM, pH 5.0). Selected samples were deconjugated using mixed glucuronidase and sulfatase from *H. pomatia* (50 and 2.3 Units, respectively) by incubation for 1 h at 37 °C, conditions that produced maximal amounts of EE2 (data not shown). Solid phase extraction was performed using 100 mg Strata C_{18}-E cartridges (Phenomenex, Torrance, CA, USA). Cartridges were activated using 1 ml acetonitrile followed by 1 ml water. Samples were then loaded onto the cartridge and washed with 1 ml 50% aqueous methanol (v/v). Samples were eluted using two aliquots of 0.5 ml methyl tert.-butyl ether–acetonitrile (20:80, v/v) and collected in glass culture tubes. The samples were evaporated to dryness at reduced pressure using a heated centrifugal concentrator. Once dry, the residues were reconstituted in 100 µl of 50% methanol–water (v/v) and were transferred to plastic vials for LC/MS/MS analysis of 90 µl injections.

2.6. Animal handling conditions

All procedures involving care and handling of rats were reviewed and approved by the NCTR Laboratory Animal Care and Use Committee. CD (Sprague–Dawley) rats were from the NCTR colony. The base diet was irradiated 5K96 meal (Purina Mills, St. Louis, MO, USA) and has been described previously [7,8].

Initial pilot studies showed that animals receiving a dietary dose of 50 ng EE2/g diet, equivalent to approximately 5 ng/kg body/day, had serum levels of EE2 below the method LOD. Another pilot study was undertaken to determine the range of blood concentrations arising from oral gavage administration of EE2 for the purposes of obtaining meaningful pharmacokinetic data. Male and female rats were removed from food and after 4 h were administered EE2 by oral gavage at 0.125–1 mg/kg body weight

using a 1 mg/ml solution of EE2 in sesame oil. The maximal amount administered was equivalent to 0.1 ml/100 g body weight. Blood was collected sequentially from the tail vein of each rat at 0.5–24 h in separator tubes, allowed to clot on ice, then centrifuged to produce serum, and aliquots were frozen at −70 °C until analysis. Control serum was similarly collected from untreated rats. The pharmacokinetic evaluation was performed using the dose of 1 mg/kg body weight because this dose produced serum EE2 concentrations sufficient to measure elimination adequately (ca. four half-lives).

2.7. Pharmacokinetic determinations

Plots of free (i.e. unconjugated) EE2 concentrations in serum as a function of time were prepared for individual rats and were analyzed using the model-independent spreadsheet approach of Ritschel [9]. Natural log-linear plots of male data showed a single phase consistent with elimination only; female data showed two phases attributed to distribution and elimination, although the distribution phase was too fast to determine the rate; no evidence for an absorption phase was observed in either sex. Approximate C_{max} values were observed at the first time point taken. The first-order elimination rate constant (k_β) was determined from the slope of the terminal phase of the ln-linear serum concentration–time curve. Internal exposure to EE2 (AUC$_{0-\infty}$, area under the time–concentration curve from zero to infinity) was estimated for individual rats using the trapezoidal rule [9]. The apparent volume of distribution for EE2 (V_d/f) was calculated as dose/(AUC × k_β) and total serum clearance was calculated as dose/AUC$_{0-\infty}$. The two-tailed t-test was used to assess statistical significance ($P < 0.05$).

3. Results

3.1. Method performance

The SPE–LC/MS/MS method described was optimized with respect to the SPE cartridge and solvents and LC column. The step gradient conditions described above were found to produce highest ES/MS/MS responses for EE2 (i.e. minimi-

zation of ion suppression and peak width) when compared to an isocratic separation (not shown). The limit of quantification (LOQ) in serum was estimated to be 2 pg on-column (signal-to-noise [S/N] ratio = 10), which corresponds to 0.03 ng/ml (0.10 nM); the detection limit (S/N ratio = 3) was approximately 0.01 ng/ml. Recovery of 1 ng/ml EE2 from serum was determined to be approximately 85% ($n=4$) based on a comparison of ^{13}C-EE2 signals from spiked serum processed through the entire method with those from a serum blank processed through the method and fortified prior to analysis. Fig. 1 shows representative chromatograms for EE2 in control and treated serum samples.

3.2. Method validation

The method was validated using control rat serum spiked with 0.1, 1, and 5 ng/ml EE2 on two separate days ($n=4$). The inter- and intra-day precision and accuracy data are shown in Table 1.

3.3. Pharmacokinetic analysis

The pharmacokinetics of ''free'' (i.e. unconjugated) EE2 were determined in rat serum. EE2 was determined to be present primarily as the unconjugated form in representative rat serum samples collected 0.5–12 h following oral gavage administra-
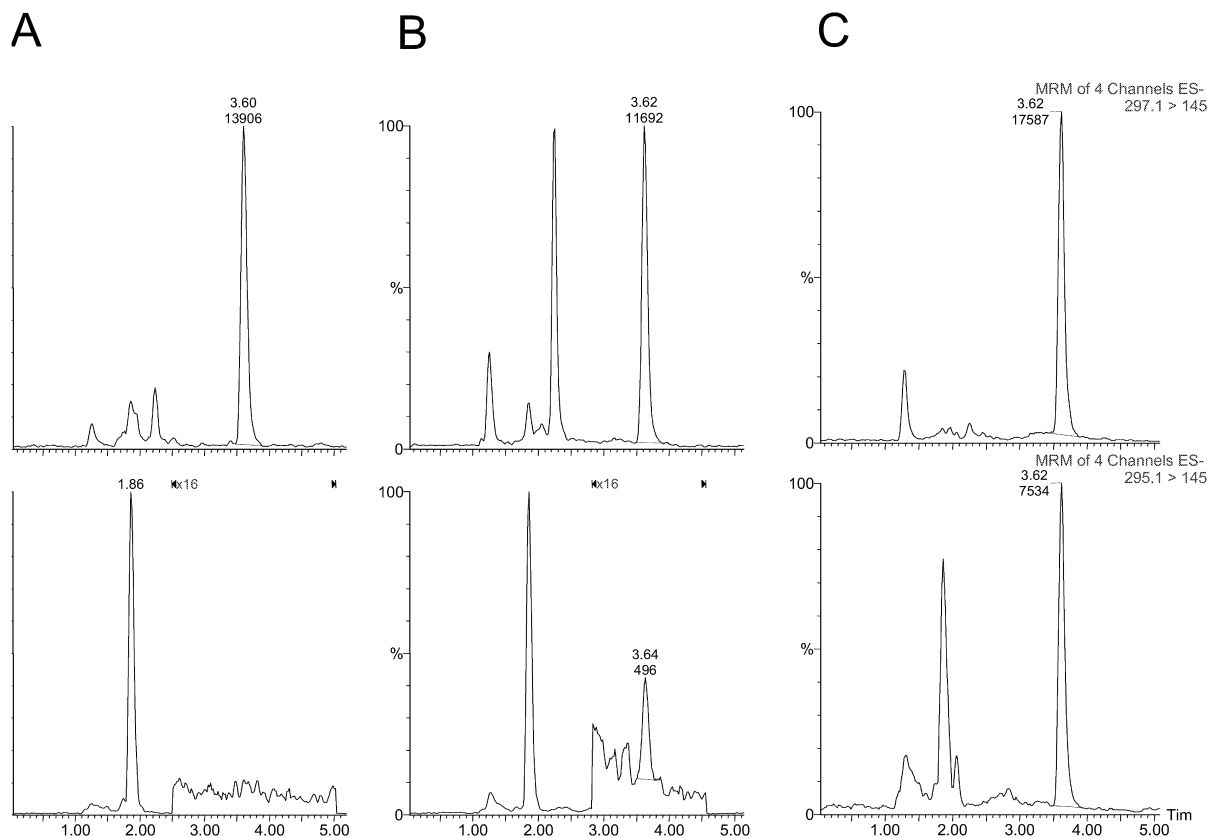

Fig. 1. Representative chromatograms for EE2 standards and incurred rat serum. The top chromatogram shows the transition for labeled EE2 internal standard (m/z 297→145) and the bottom chromatograms show the transition for unlabeled EE2 (m/z 295→145) for: (A) serum from an untreated rat (75 µl) containing 100 pg internal standard; (B) rat serum collected 12 h after administration of an oral gavage dose of EE2 (0.06 ng/ml); (C) rat serum collected 0.5 h after administration of an oral gavage dose of EE2 (0.65 ng/ml). Note: the retention time in min and the integrated peak area are listed above the EE2 peaks.

Table 1
Intra- and inter-day precision and accuracy for LC–ES/MS/MS analysis of EE2 in rat serum

EE2 added (ng/ml)	EE2 measured		Accuracy (%)
	Day 1	Day 2	
0	<LOD	<LOD	–
0.1	0.089±0.008 (9%)	0.089±0.004 (5%)	89
1	0.93±0.064 (7%)	0.92±0.029 (3%)	93
5	4.7±0.085 (2%)	4.7±0.11 (2%)	94

Serum from untreated rats was spiked with different amounts of EE2 and analyzed on separate days as described in Section 2 ($n=4$).

tion by comparing concentrations before and after treatment with a mixture of *H. pomatia* glucuronidase/sulfatase enzymes. The serum concentrations of free and conjugated EE2 were determined at all time points for a single female rat. The concentration of conjugated EE2 decreased rapidly at early time points (0.5–2 h) to low levels (0.2 ng/ml) and as a result, the percentage of unconjugated EE2 increased from 43% at 0.5 h to 85% at 2–4 h (data not shown).

A preliminary dose range-finding study was performed to determine the relationship of EE2 C_{max} with administered gavage dose. Over the dose range of 0.125–1.0 mg/kg body weight, C_{max} was determined at 30 min in individual female rats and showed a linear increase from 0.04 to 0.48 ng/ml (slope=0.50 ng/ml per mg/kg body weight, correlation coefficient 0.998, $n=4$ doses).

Model-independent pharmacokinetic analysis was performed using sequential blood sampling from individual male and female rats following administration of EE2 at 1 mg/kg body weight. Fig. 2 shows representative ln-transformed plots of EE2 concentration vs. time for a male and female rat. Tables 2 and 3 show the individual and average pharmacokinetic parameters for all male and female rats, respectively. Several significant sex differences were found for the average pharmacokinetic parameters between males and females: while a distribution process in males was not observed in males (see Fig. 2), distribution was apparent in the females, albeit too rapid to quantify; the average elimination rate constant (k_β) was 2.3-fold slower for females (see Fig. 2); and the apparent volume of distribution (V_d/f) was 2.4-fold greater for females. However, the C_{max}, $AUC_{0-\infty}$, and the total serum clearance values were comparable.

4. Discussion

This article describes a new method for high sensitivity analysis of unconjugated EE2 (LOQ 0.10 nM from 75 µl serum) that used electrospray in conjunction with isotope dilution tandem mass spectrometry for quantitative measurements. The performance of this method was comparable to that recently reported by Anari et al. [10] for LC–ES/MS/MS analysis of EE2 in monkey plasma following dansyl chloride derivatization (LOQ 0.02 nM). However, even with this degree of analytical sensitivity, the high estrogenic potency of dietary EE2 led to undetectable serum levels in diet-exposed rats (2, 10, 50 ng EE2/g diet). These diet compositions were selected to encompass the range for women
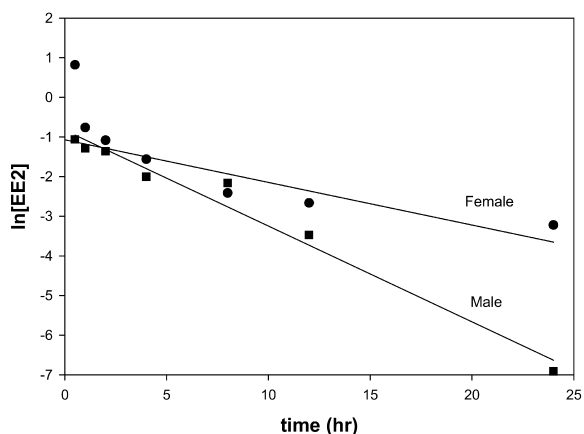

Fig. 2. Representative ln transformation plots of free EE2 (ng/ml) serum pharmacokinetics for a representative male (#6, squares) and female (#12, circles) rats treated with a dose of 1 mg/kg. The linear relationships are shown for the terminal elimination phases.

Table 2
Compilation of pharmacokinetic parameters for a 1-mg/kg oral gavage dose of free EE2 in male Sprague–Dawley rats

PK parameter	M1	M2	M3	M4	M5	M6	Mean±SD
$t_{1/2}$ (h)	3.04	3.11	2.11	2.70	2.57	3.01	2.76±0.38*
$AUC_{0-\infty}$ (ng/h/ml)	5.26	2.96	2.94	2.10	2.31	1.89	2.91±1.23
V_d/f (l/kg)	830	1510	1040	1850	1600	2300	1520±530*
C_{max} (ng/ml)	0.46	1.37	1.29	0.48	0.60	0.35	0.76±0.45
Cl_{tot} (ml/kg/h)	190	340	340	480	430	530	380±120

The pharmacokinetic parameters from serum concentration data ($n = 6$) were determined using model-independent analysis and statistical significance was assessed using the two-tailed t-test. Correlation coefficients for the individual ln transformed plots ranged from 0.82 to 0.98.
* $P < 0.05$ for significant sex difference.

using oral contraceptives where doses are less than 1 µg/kg body weight and are based on effects observed in adults from a dose range-finding study (Delclos, unpublished data). In order to obtain pharmacokinetic information about EE2 and possible sex-specific differences, we used oral gavage dosing at 1 mg/kg body weight to achieve higher circulating levels, yet maintain the effects of absorption from the gut and first-pass metabolism in the gut and liver [11]. Oral gavage with different amounts of EE2 produced a linear increase in C_{max} suggesting that saturation of absorption and metabolism did not occur over this dose range (0.125–1 mg/kg).

Significant pharmacokinetic differences in male rats relative to females were observed (see Tables 2 and 3) for elimination (faster) and the apparent volume of distribution (smaller); however, several other important parameters (C_{max}, $AUC_{0-\infty}$, and total serum clearance) showed no significant sex differences, in part because of inter-animal variability. Values for the apparent V_d/f, although appearing quite high, are comparable to that determined in

female Wistar rats following intravenous administration (73 l/kg) when considering the very low degree of oral bioavailability observed in the monkey ($f = 0.009$) [10] and rat ($f = 0.03$) [12]. The low degree of bioavailability is likely the result of extensive metabolism, both in the gut and the liver [12]. The sex differences in both elimination half-life and V_d/f could be the result of differences in metabolism. Alternatively, such a large V_d/f is consistent with extensive distribution into tissues and protein binding and the significantly higher V_d/f in females suggests that estrogen receptor binding may contribute.

EE2 is the third environmental estrogenic compound tested in Sprague–Dawley rats under similar conditions to determine possible developmental, reproductive, and chronic toxicity. Issues surrounding metabolism and disposition were important components in these studies on genistein, *p*-nonylphenol, and EE2. Extensive conjugation of the phenolic groups were observed in rat serum for genistein (97–99%, [7]) and *p*-nonylphenol (95–97%, [8]) following oral administration; however, EE2 was

Table 3
Compilation of pharmacokinetic parameters for a 1-mg/kg oral gavage dose of free EE2 in female Sprague–Dawley rats

PK parameter	F1	F2	F3	F4	F5	F6	Mean±SD
$t_{1/2}$ (h)	2.39	9.90	6.08	7.14	4.53	6.73	6.13±2.54*
$AUC_{0-\infty}$ (ng/h/ml)	1.0	2.52	2.1	2.61	3.01	4.17	2.57±1.04
V_d/f (l/kg)	3450	5670	4180	3950	2170	2330	3620±1300*
C_{max} (ng/ml)	0.32	0.64	0.74	1.22	1.41	2.28	1.10±0.70
Cl_{tot} (ml/kg/h)	1000	397	476	383	332	240	470±270

The pharmacokinetic parameters from serum concentration data ($n = 6$) were determined using model-independent analysis and statistical significance was assessed using the two-tailed t-test. Correlation coefficients for the individual ln transformed plots ranged from 0.74 to 0.99.
* $P < 0.05$ for significant sex difference.

conjugated to a much lower extent at a similar time (57% at 0.5 h). This finding is consistent with the low degree of estradiol conjugation reported previously (18%, [13]). It was previously observed for phenolic xenoestrogens that the conjugated forms in rats [8,14] and in humans [15] were almost exclusively glucuronides; however, EE2 sulfates and glucuronides are found in comparable amounts in baboons [16] and monkeys [17]. This difference in enzymatic conjugation may be related in part to the much lower serum concentrations of EE2 observed (<4 nM) relative to genistein (<10 µM, [7]) and p-nonylphenol (<1 µM, [8]) because K_m values for xenobiotic phenolic compounds are typically observed in the range of 10–1000 µM for UDP glucuronosyl transferases [15,18] and sulfotransferases [15]. For EE2, genistein, and p-nonylphenol, the elimination half-lives were similar among groups of either male or female rats; however, significant sex differences in half-lives were observed for females (6.1, 4.3, and 4.0 h, respectively) relative to males (2.8, 3.0, and 3.1 h, respectively) in that females eliminated all three estrogens slower than did the males. The corresponding AUC values for genistein and p-nonylphenol were significantly larger for female rats relative to males, but this finding was reversed for EE2, possibly as a result of the high female V_d. These pharmacokinetic comparisons of EE2, genistein, and p-nonylphenol will be useful for interpreting treatment-specific effects of these environmental estrogens in rodent studies of endocrine disruption currently nearing completion at this institution.

Acknowledgements

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