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Derivatization of Ethinylestradiol with Dansyl Chloride To Enhance Electrospray Ionization: Application in Trace Analysis of Ethinylestradiol in Rhesus Monkey Plasma

M. Reza Anari,* Ray Bakhtiar, Bing Zhu, Su Huskey, Ronald B. Franklin, and David C. Evans

Preclinical Drug Metabolism, Merck Research Laboratories, RY80L-109, Rahway, New Jersey 07065

The extensive metabolism and administration of low doses of ethinylestradiol (EE) in preclinical animal species necessitates a sensitive analytical method to quantify the drug at low picogram-per-milliliter concentrations in biological matrixes. A highly sensitive and accurate method based on the derivatization of EE with dansyl chloride coupled with liquid chromatography/tandem mass spectrometry is described. The dansyl derivatization of EE introduced a basic secondary nitrogen into the molecule that was readily ionized in commonly used acidic HPLC mobile phases. The derivative showed an intense protonated molecular ion at *m*/*z* 530 under positive turbo ion spray ionization. The collision-induced dissociation of this ion formed a distinctive product at *m*/*z* 171, corresponding to the protonated 5-(dimethylamino)naphthalene moiety. The selected reaction monitoring, based on the *m*/*z* 530 → 171 transition, was highly specific for EE, since no background signal was observed from blank plasma obtained from rhesus monkeys. The limit of detection, at a signal-to-noise ratio of 5, was 0.2 fg/mL EE spiked into blank plasma. This allowed for a lower limit of quantitation of 5 pg/mL using a 50-μL plasma sample and 10-μL injection of dansylated derivative into the CTC−PAL Leap autosampler coupled to a Sciex API 4000 mass spectrometer. Using fast-gradient liquid chromatography, the analyte peak eluted at 1.6 min. The validation results showed high accuracy (% bias < 4) and precision (% CV < 7.5) at broad linear dynamic ranges (0.005−20 ng/mL), using deuterated EE as internal standard. Therefore, the facile dansyl derivatization coupled with tandem mass spectral analysis allowed the development of a highly sensitive and specific method for quantitation of trace levels of EE in the plasma of rhesus monkeys dosed orally and intravenously with EE.

Ethinylestradiol (EE) is a potent, synthetic estrogen that has been used worldwide as a major component of combined oral contraceptive preparations. With the introduction of the low-dose preparation of EE (30−50 μg EE/tablet/day) there has been a growing concern about the possible interaction of other drugs with EE and a potential failure of contraception in women taking oral contraceptives.[1] Inducers of cytochrome P450 isoforms, such as the antituberculosis drug, rifampicin;[2] anticonvulsants, such as phenobarbital,[3] phenytoin,[4] and carbamazepine;[4] as well as antibiotics, such as ampicillin[5] and griseofulvin,[6] have been reported to increase the incidence of breakthrough bleeding and unwanted pregnancies in women using oral contraceptives.[7] The enhanced clearance of EE due to the induction of drug-metabolizing enzymes, especially cytochrome P450 isoform CYP3A4,[8,9] has been suggested as a major mechanism for EE−drug interactions.[10] Human CYP3A4 is involved in the biotransformation and clearance of >50% of current drugs as well as new chemical entities.[11] Therefore, it is imperative to examine the potential interaction of investigational new drugs with EE during the course of drug development, especially those that show an affinity toward CYP3A4.[12−18] Early studies in animal models that mimic the human in vivo EE−drug interaction can help in the design of drugs that may be used safely in women taking oral contraceptives. To address the pharmacokinetics and potential oral contraceptive interactions in preclinical species, it is essential to have a highly

* Corresponding author. Telephone: 732-594-6184. Fax: 732-594-4390. E-mail: reza_anari@merck.com.

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sensitive analytical method that can accurately measure trace levels of EE in small volumes of plasma.

Assay methods, such as gas chromatography/mass spectrometry (GC/MS), have been used for analysis of EE.[19] However, a radioimmunoassay (RIA) that has a lower limit of quantitation of 8 pg/mL has been used widely as the method of choice.[20,21] Although the RIA offers the highest sensitivity, the method suffers from a lack of specificity as a result of cross reactivity of EE metabolites and progestins with the antibody. This may, in turn, lead to an overestimation of EE, and the assay has produced conflicting results, with estimated peak plasma concentrations varying significantly between a low of 40 pg/mL and a high of 2100 pg/mL following an oral dose of 50 μg to humans.[22,23] Even with the introduction of monoclonal antibodies, the separation of EE from interfering plasma components is essential to achieve an accuracy of 80−120%.[24] Thus, use of combined solid-phase extraction and liquid chromatography prior to RIA is necessary to separate steroids and polar metabolites that may lead to cross-reactivity.[25,26] The commercially available radioimmunoassays that have incorporated such clean up steps (e.g., Ridascreen, Accurate Chemical & Scientific Corp.) exhibit 0.7% cross-reactivity. Although solid-phase extraction and HPLC purification minimize the cross-reactivity problem inherent with RIA, the required time for preparation and analysis of 10 samples is ∼7 h. This low throughput limits the application of RIA to the analysis of large number of samples from drug−drug interaction studies. Moreover, the RIA, as well as the GC/MS assay, requires more than 0.5 mL of blood or serum,[21,24,26] making these assays impractical for pharmacokinetic studies in preclinical species.

A number of other classical assays based on the fluorometric or chemiluminescence detection of dansyl derivatized estrogens have been reported.[27−30] The fluorescence is not related to EE per se, but rather originates from the dansyl moiety. Therefore, other endogenous estrogens and their metabolites that are coderivatized, are detected simultaneously.[27] Even if all of the interfering analytes are separated by using a longer chromatographic separation, the nanogram detection limit reported for EE is higher than the peak plasma concentration of the drug in humans and preclinical species.[31]

The use of tandem mass spectrometry (MS/MS) as a highly specific detection technique for EE could resolve the issue of interference from steroidogenic plasma components. The high specificity of MS/MS allows for the discrimination and quantitation of analytes from coeluting metabolites or from a biological matrix without the need for long chromatographic separation.[32] In this regard, new methods for the determination of EE in wastewater that are based on the immunoaffinity or solid-phase extraction coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) have been described.[33,34] Although the authors report nanograms-per-liter detection limit, these assays require liters of wastewater samples to be concentrated on the solid phase or immunoaffinity cartridges, a procedure that is not practical for analysis of small amounts of biological samples. Furthermore, despite higher specificity, the application of tandem mass spectrometry for analysis of EE is limited as a result of the low ionization and detection using current atmospheric pressure ionization techniques.

The objective of the present study was to develop a sensitive and accurate method that could be used to analyze trace picogram concentrations of EE from small amounts of plasma (typically 50−100 μL) from preclinical species. We sought to use a facile aqueous phase derivatization procedure in order to introduce a highly ionizable moiety into the EE core structure. The simulated ionization efficiency of a number of feasible derivatives with the 3- or 17-hydroxyl functional groups indicated that the addition of a dansyl moiety should enhance the ionization of EE significantly. The strong signal of 3-dansyl-EE under positive turbo ion spray mass spectrometry allowed for the development of a highly sensitive and selective LC/MS/MS method for the analysis of EE using small amounts of plasma from rhesus monkeys dosed with EE.

EXPERIMENTAL SECTION

Chemicals. Ethinylestradiol (17α-ethynyl-1,3,5(10)-estratriene-3,17β-diol) was purchased from Steraloids, Inc. (Newport, RI). The internal standard, 17α-ethinylestradiol-2,4,16,16-d_4 (D_4-EE), was obtained from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada). Dansyl chloride (5-(dimethylamino)-1-naphthalenesulfonyl chloride), with ≥99.0% HPLC purity, was obtained from Sigma-Aldrich (St. Louis, MO). HPLC-grade acetonitrile was obtained from J. T. Baker (Phillipsburg, PA). Formic acid was purchased from GFS Chemicals (Columbus, OH), and HPLC-grade water was obtained from Mallinckrodt Baker, Inc. (Paris, KY). Ethyl acetate (ACS certified), methanol, and reagent-grade sodium hydrogen carbonate were obtained from Fisher Scientific (Pittsburgh, PA). The drug-free (blank) rhesus monkey plasma containing EDTA as anticoagulant was obtained from Bioreclamation Inc. (Hicksville, NY).

Ethinylestradiol Extraction and Derivatization. Aliquots (50 μL) of plasma were diluted with 0.5 mL of water, followed by the addition of 50 μL of D_4-EE (0.1 μg/mL in 10% methanol) as the internal standard. Samples were mixed thoroughly, followed by addition of 2 mL of ethyl acetate to each test tube. The samples

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were vortex-mixed and the organic phase (1.5 mL) was transferred to another set of clean test tubes and evaporated to dryness under a stream of nitrogen using TurboVap II (Zymark, MA). The residue in each tube was redissolved in 100 μL of sodium bicarbonate buffer (100 mM, pH adjusted to 10.5 with NaOH) followed by vortex-mixing for 1 min. To each sample, 100 μL of dansyl chloride solution (1.0 mg/mL in acetone) was added followed by vortex-mixing for another 1 min. The samples were placed in a 60 °C incubator for ~3 min, then cooled to room temperature and transferred to 250-μL conical vials for MS analysis.

Estimation of the Apparent Dissociation Constant and Ionization Efficiency. The apparent dissociation constant (pK_a) was estimated using the ACD/pK_a DB (Advanced Chemistry Development, Toronto, Canada) software, which calculates accurate acid−base ionization constants under standard conditions at 25 °C and zero ionic strength in aqueous solutions. Each calculation is expressed with its 95% confidence limits, considering both the substituent constants and the inductive electronic effects. Percent ionizations were calculated using eqs 1 and 2 for the acidic and basic functional groups, respectively.

$$\% \text{ ionization} = \frac{100}{1 + 10^{(\text{pKa}-\text{pH})}} \quad (1)$$

$$\% \text{ ionization} = \frac{100}{1 + 10^{(\text{pH}-\text{pKa})}} \quad (2)$$

The pK_a and percent ionization values were estimated for the acid form of the 3-hydroxy and 17-hydroxy functional groups in EE and for 17-hydroxyl group in 3-dansyl-EE. For the 5-(dimethylamino)naphthalene-1-sulfonate substitution in 3-dansyl-EE, the pK_a was derived from the conjugated acid form of the basic amine. The percent ionization equation suitable for basic drugs (eq 2) was used for the 5-(dimethylamino)naphthalene-1-sulfonate moiety.

High Performance Liquid Chromatography. The HPLC system consisted of a Leap Technologies HTS CTC-PAL autosampler (Carrboro, NC) and two Perkin-Elmer series 200 micropumps (Norwalk, CT). The fast gradient chromatographic separation was carried out using a Phenomenex Synergi 4μ Max-RP (2 × 50 mm, 4μ, 80 Å) column (Torrance, CA). The mobile phase flowing through the column consisted of two eluants, solvent A (95% H_2O/5% acetonitrile, 0.1% formic acid, v/v) and solvent B (95% acetonitrile/5% H_2O, 0.1% formic acid, v/v) with a flow rate of 400 μL/min. The column was maintained at initial conditions of 50% B for 6 s, followed by a linear fast gradient to 95% B over 24 s. This condition was maintained for 66 s, then returned to the initial conditions over 36 s and maintained until the end of a 3-min run. The CTC-PAL Leap cooling unit was set at 10 °C. The volume of injection onto the column was 10 μL, and the injector syringe was prewashed (100 μL) with water twice prior to each injection. The syringe, injection loop, and the switching valve were postwashed (100 μL) five times by two wash solutions sequentially. The first wash solution was water, and the second wash solution was acetonitrile containing formic acid (0.1%, v/v).

Mass Spectrometry. The analytes were detected using a PE Sciex API 4000 (Toronto, Canada) triple quadrupole mass spectrometer equipped with a turbo ion spray source operated in the positive selected reaction monitoring (SRM) mode. All of the source and instrument parameters were optimized by infusing the 3-dansyl-EE or 3-dansyl-D_4-EE, which were isolated from an HPLC column using fluorometric detection,[27] into the mass spectrometer. The purified derivatives were infused at 10 μL/min by an infusion pump (Harvard Apparatus, South Natick, MA) teed into the mobile-phase mixture (50%) at a flow rate of 400 μL/min. Both Q1 and Q3 were operated under unit mass resolution (0.7 Da at full width half-maximum). Nitrogen was used as the curtain (setting 10), drying gas 1, (setting 50), drying gas 2 (setting 50), and collision gas (setting 6), which was delivered from a nitrogen Dewar with the gas regulator maintained at 80 psi. The electrospray voltage was set at 4500 V, and the turbo ion spray interface was maintained at 500 °C. The specific SRM transitions for 3-dansyl-EE and 3-dansyl-D_4-EE (internal standard) were monitored (200 ms dwell time/transition) at m/z 530 → 171 and 534 → 171, respectively. The declustering potential, collision energy, and collision exit potentials were optimum at 140, 51, and 14 V for the analyte and internal standard, respectively. The data were collected and quantified using PE Sciex Analyst software (version 1.2).

Method Validation. The analytical method was validated to demonstrate the specificity, recovery, limit of detection (LOD), limit of quantitation (LOQ), precision, and accuracy of samples. Triplicate sets of standard curve and quality control (QC) samples were analyzed on 3 separate days to determine the inter- and intraday validation. The minimum acceptance criteria are described below, which are similar to the guidelines set by FDA for bioanalytical method validation.

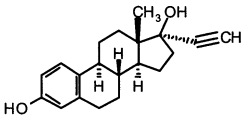
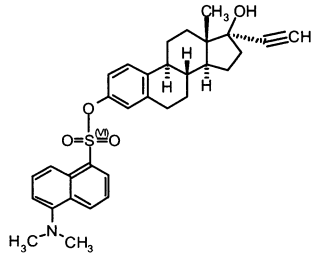
(a) Specificity. Acceptable specificity was defined as the absence of any detectable SRM LC/MS/MS ion currents at the retention time regions of 3-dansyl EE and 3-dansyl-D_4-EE in blank plasma samples (double blanks).

(b) Recovery. The recovery was determined by comparing the preextraction spike to the postextraction spikes for three replicates of the high-, medium-, and low-level quality control (QC) samples. For the postextraction spike, the QC analytes were spiked into the sample extract and mixed immediately prior to evaporation under nitrogen.

(c) Method Linearity. The calibration range for EE was established using triplicate set of standards from 0.005 to 20 ng/mL EE in blank rhesus monkey plasma. The standard concentrations were 0.005, 0.010, 0.025, 0.05, 0.100, 0.250, 0.500, 1.00, 5.00, and 20.00 ng/mL. The intra- and interassay mean accuracy (% bias) of the calibration curve standards were within ±15% of the expected concentration (20% at the LOQ). The inter- and intraassay precision (coefficient of variation) of the calibration curve standards were within ±15% (20% at the LOQ). The accepted criterion for linearity was a coefficient of determination (R^2) of >0.98, using at least six sets of standard concentrations. The combined triplicate set of standards, based on a linear regression and $1/X^2$ weighting, was used in all experiments for the quantification of samples and QCs.

(d) Precision and Accuracy. Twelve replicates (n = 12) at 0.010, 0.050, 0.500, and 5.00 ng/mL were used to make the low- to high-range QC concentrations. The target values for intra- and interassay mean accuracy and precision of each QC sample were less than ±15% of the expected concentration.

Table 1. Predicted pK_a and Percent Ionization for Various Functional Groups in Ethinylestradiol and 3-Dansyl-ethinylestradiol

Chemical Structure	Functional Group	pKa [a]	% Ionization [b]	
			pH 3.0	pH 8.0
 Ethinylestradiol	3-OH	10.4 ± 0.6	< 0.001	0.397
	17-OH	13.3 ± 0.4	< 0.001	< 0.001
 3-Dansyl-ethinylestradiol	3-O-Dansyl	3.3 ± 0.4	67.1	0.002
	17-OH	13.1 ± 0.4	< 0.001	< 0.001

[a] The apparent pK_a ± 95% confidence limits was estimated using the ACD/pK_a DB software as described in the Experimental Section. The pK_a for the 3-hydroxy in EE and the 17-hydroxy in EE and 3-dansyl-EE was estimated for its acid form. The pK_a for the 5-(dimethylamino)naphthalene-1-sulfonate (dansyl) moiety at the 3-O position was estimated for the conjugated acid of the basic amine form (pK_b ~ 10.7). [b] The percent ionizations were calculated using the equations described in the Experimental Section.

Pharmacokinetic Analysis. Four female rhesus monkeys were dosed intravenously (i.v.) at 0.01 mg/kg EE using a vehicle of ethanol/propylene glycol/water (20:30:50, v/v/v). At a later date, the same four monkeys were administered a single oral (p.o.) dose of EE at 0.3 mg/kg using a vehicle of 0.5% methylcellulose containing 0.02% sodium lauryl sulfate. Blood samples were collected into tubes containing EDTA as the anticoagulant, at times 0.083 (for IV only), 0.25, 0.30, 1, 2, 4, 6, 8, and 24 h post-EE administration. Plasma was separated from whole blood by centrifugation at 4 °C, and samples were stored at − 70 °C until analysis. The area under the plasma ethinylestradiol concentration versus time curve over the first 8 h of dosing (AUC_{0-8hr}) was estimated using a linear trapezoidal approximation. The total plasma clearance (CLp) was estimated from the equation CLp = dose/AUC, and the volume of distribution at steady state (Vdss) was calculated from the equation Vdss = CLp × mean residence time. For the estimation of terminal half-life ($t_{1/2}$), regression analysis of the plasma concentrations of EE was used at the 4-, 6-, and 8-hr time points. The absolute oral bioavailability (F) was calculated using the equation: F = AUC (p.o.) × dose (i.v.)/AUC (i.v.) × dose (p.o.).

RESULTS AND DISCUSSION

Recent advances in the development of atmospheric pressure ionization (API) mass spectrometry have allowed for the specific detection of a wide array of biochemicals at low concentrations within endogenous matrixes.[32] However, direct analysis of EE by current API ion sources has not resulted in a low quantitation limit. The lowest detection limit (S/N ≥ 5) achieved for EE in our laboratory was >50 ng/mL using negative atmospheric pressure chemical ionization. This value was higher than EE concentrations reported in plasma (0.050−10 ng/mL) from human and preclinical species.[31,35] The estrogenic steroid EE possesses the hydrophobic aromatic steroid core structure as well as the two hydroxyl functional groups common to estradiols. Our simulation of the dissociation constants (pK_a) indicated that the phenolic and alcoholic hydroxyl groups conferred estimated pK_a values of 10.4 and 13.3 to the molecule, respectively (Table 1). The estimation of ionization efficiency revealed that, under acidic to neutral pH ranges suitable for robust liquid chromatographic separations, underivatized EE was ionized minimally in solution (Table 1). This may explain, in part, the low ionization of EE using electrospray, since ionization in the gas phase is known to be directly affected by ionization in solution.[36] In addition, we observed that the collision-induced dissociation of EE did not generate an intense fragment ion that could be used for specific detection using selected reaction monitoring tandem mass spectrometry. On the basis of our preliminary studies, the ratio of the intensity of fragment ion to parent molecule was <1.4% for EE, whereas for other nonaromatized steroids, a ratio >20% was achievable (data not shown).

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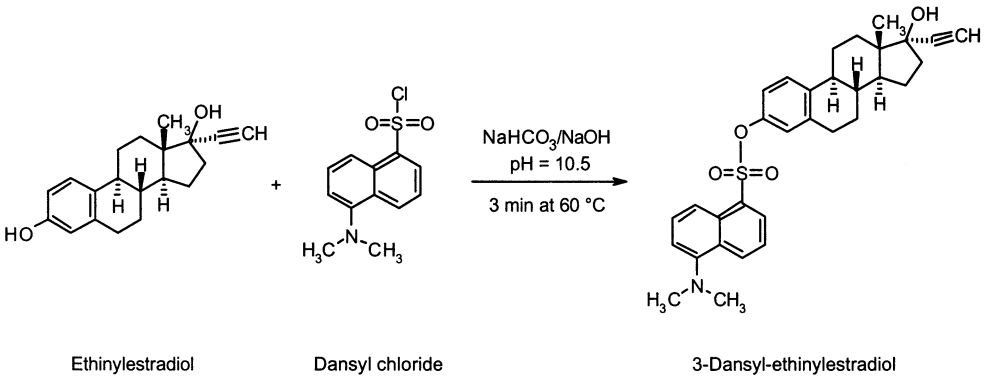

Figure 1. Schematic representation for the aqueous-phase chemical derivatization of ethinylestradiol by dansyl chloride.

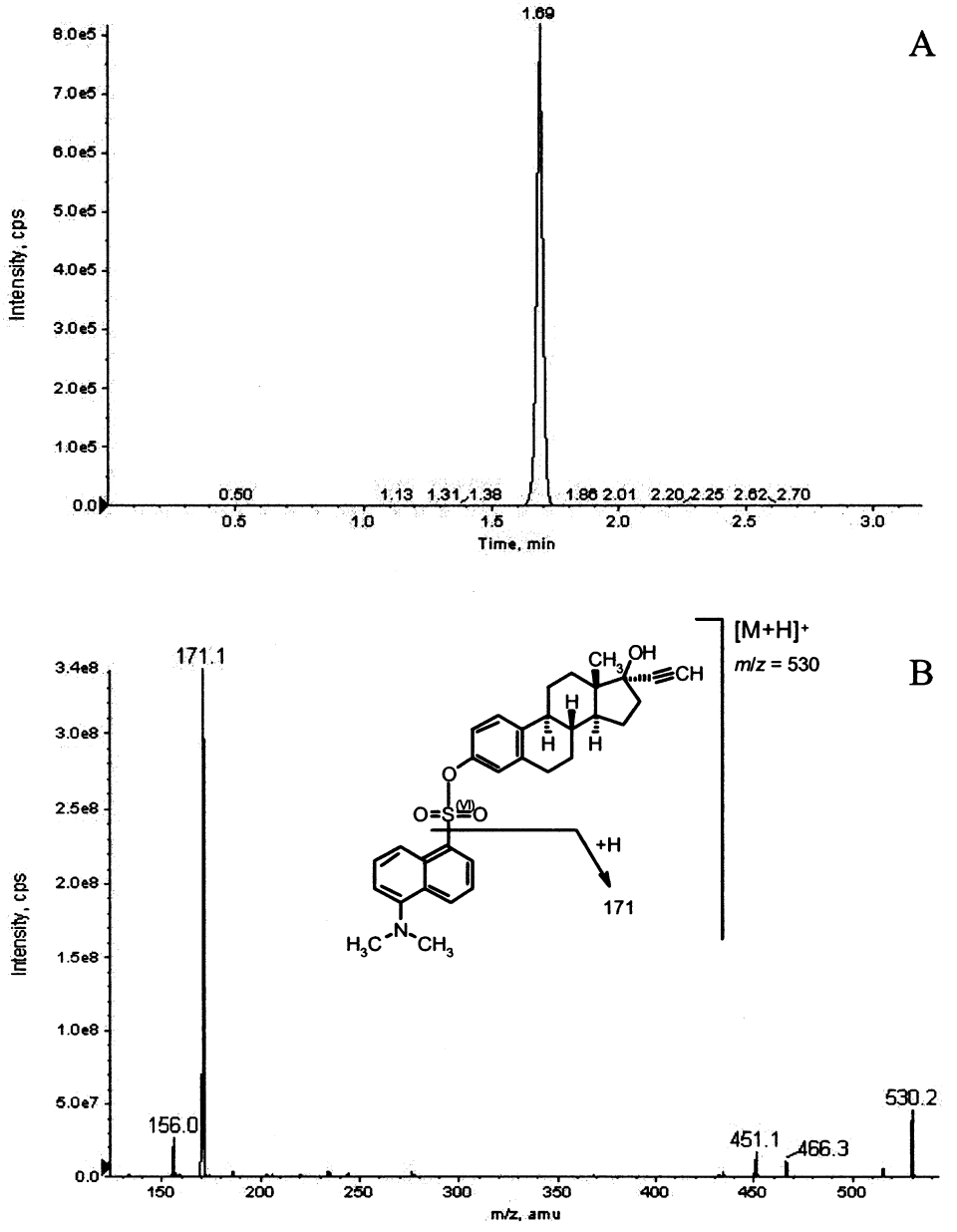

Figure 2. Reconstructed ion chromatogram and product ion scan of the isolated 3-dansyl-ethinylestradiol. (A) Reconstructed ion chromatogram of *m*/*z* 529.5−530.5 from the Q1 full scan range from 300 to 600 amu under +ESI. Plasma samples spiked with EE (5 ng/mL) were extracted and derivatized as described in the Experimental Section, and 10 μL was loaded into the column. (B) Product ion scan of the *m*/*z* 530 at collision energy of 51 eV under optimum source parameters is shown as described in the Experimental Section.

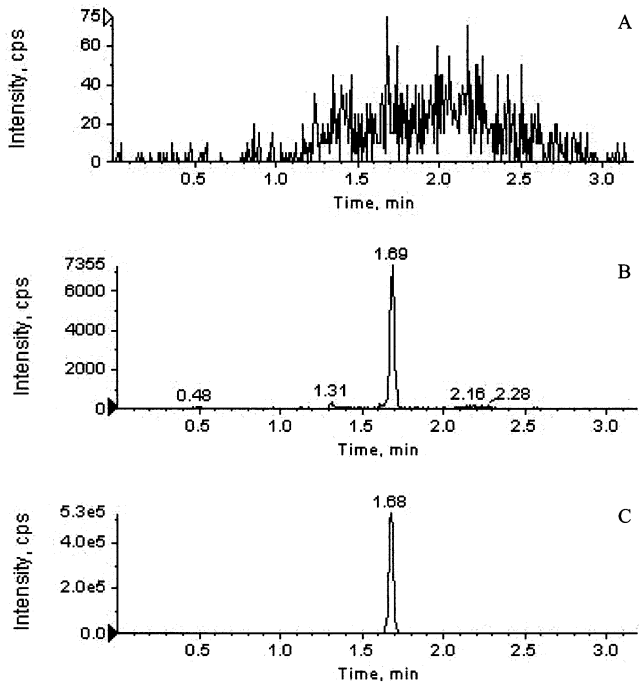

Figure 3. Reconstructed ion chromatogram for 3-dansyl-ethinylestradiol and 3-dansyl-D_4-ethinylestradiol specific SRM transitions from blank and spiked monkey plasma. The LC/MS/MS of the SRM transitions for 3-dansyl-ethinylestradiol and 3-dansyl-D_4-ethinylestradiol in actual samples were described in the Experimental Section. (A) Reconstructed ion chromatogram for the MRM transition *m*/*z* 530 → 171 following extraction and derivatization of 50 μL of blank female monkey plasma (10-μL injection volume). (B) Reconstructed ion chromatogram for the MRM transition *m*/*z* 530 → 171 following extraction and derivatization of 50 μL of blank female monkey plasma spiked with 5 pg/mL EE (10-μL injection volume). (C) Reconstructed ion chromatogram for the specific MRM Transition at *m*/*z* 534 → 171 following extraction and derivatization of 50 μL of blank female monkey plasma spiked with 5 ng/mL D_4-ethinylestradiol (10-μL injection volume).

In light of the low ionization and fragment efficiency of the EE core structure, we sought to introduce a highly ionizable group into the molecule using a facile aqueous phase derivatization reaction.[37,38] Our simulation of the ionization efficiency for a number of feasible derivatives indicated that the addition of a dansyl moiety should enhance the ionization of EE significantly (Table 1). The derivatization of phenolic groups with dansyl chloride is a rapid, aqueous-phase, chemical reaction that has been used widely for the fluorometric detection of estrogens.[27,30,39] The introduction of a dansyl functional group bearing a basic nitrogen shifted the predicted pK_a of the molecule to 3.3 ± 0.4 (pK_a of the conjugated acid form of the basic amine), which as we postulated, should significantly enhance ionization under acidic conditions (for example, mobile phases containing 0.1% formic acid, pH 3) (Table 1).

The details of the EE extraction and derivatization conditions have been described in the Experimental Section. Under optimum conditions of extraction and derivatization, recovery of EE was >95%, and the derivatization yield was ~92% (data not shown), similar to previously published estimates.[27,39] Figure 1 shows the derivatization reaction scheme that reached a high constant yield within 3 min.[27] The ionization and fragmentation of the isolated dansyl derivative was studied using electrospray tandem mass spectrometry. As expected, the 3-dansyl-EE produced an intense molecular ion peak at *m*/*z* 530 under positive turbo ion spray (Figure 2A). The collision-induced dissociation spectra of *m*/*z* 530 produced an intense fragment ion at *m*/*z* 171 corresponding to the 5-(dimethylamino)-naphthalene moiety (Figure 2B). This pattern of fragmentation is similar to that of other amino acid and phenolic dansyl derivatives.[40,41] Using electron ionization mass spectrometry, a modified McLafferty rearrangement mechanism gave rise to the product ion at *m*/*z* 171, the characteristic fragment ion from cleavage of the C−S bond, followed by the rearrangement of one hydrogen atom toward the naphthalene nucleus.[40] The product ion at *m*/*z* 170, which is the result of direct C−S cleavage without hydrogen rearrangement,[40,42] accounted for 20% of the intensity of the *m*/*z* 171 ion. The intensity of the product ion at *m*/*z* 171 was 600% greater than the protonated parent ion at *m*/*z* 530 (Figure 2B) under optimum collision energy at 51 eV. This is a significant improvement over the fragmentation efficiency of underivatized EE (1.4% using *m*/*z* 295 → 145 transition under negative electrospray ionization; unpublished observation).

The selected reaction monitoring, based on the *m*/*z* 530 → 171 transition, was specific for EE: there was no detectable signal from the blank plasma from the female rhesus monkeys using this transition (Figure 3A). Although other endogenous estrogens present in female monkey plasma were presumably coextracted and derivatized by dansyl chloride,[27] no interference was observed due to the highly specific parent mass-to-charge ratio and the characteristic product ion used in the *m*/*z* 530 → 171 transition. Therefore, the fast gradient liquid chromatographic approach was used to elute the dansylated- EE and D_4-EE at 1.69 and 1.68 min, respectively (Figure 3B and 3C). The retention times, as well as peak shapes, were remarkably stable over the course of validation and pharmacokinetic studies following 300 injections (data not shown).

On the basis of the signal-to-noise ratio of 5, the limit of detection (LOD) for EE was ~0.2 fg/mL. This LOD was achieved by extraction of 50-μL plasma samples and injection of 10-μL derivatized product (>100 μL derivatized product/sample). The LOD reported for the two separate validated RIA procedures ranged from 3 to 5 pg/mL, using 0.3−0.5 mL human serum.[21] The LOD for a validated GC/MS assay was ~10 pg/mL when 1 mL of human serum was used.[21] In the present LC/MS/MS assay, the signal-to-noise ratio was >100-fold at the LOQ, that is, 5 pg/mL when EE was spiked into blank plasma (Figure 3B). This suggested that the method may be capable of attaining an even lower LOQ were it not for the carryover problem. This problem is encountered commonly during trace analysis and, in this instance, was addressed successfully by using a CTC-PAL autosampler equipped with a dual washing system. With dual,

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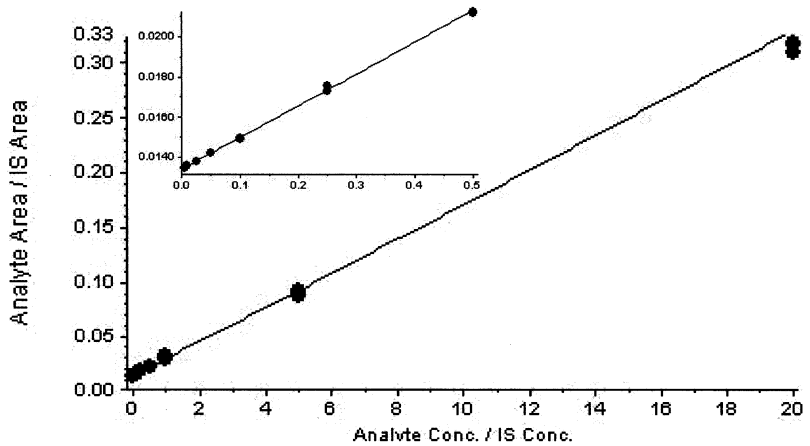

Figure 4. Triplicate calibration standard curve for ethinylestradiol spiked into rhesus monkey plasma. Three sets of female rhesus monkey plasma samples were spiked with 0.005−20 ng/mL ethinylestradiol. The samples were extracted and derivatized by dansyl chloride after addition of D_4-EE as the internal standard (IS). The SRM transitions at *m*/*z* 530 → 171 and 534 → 171 were monitored under positive turbo ion spray for 3-dansyl-EE and D_4-3-dansyl-EE, respectively. The ratio of area under curve for EE to D_4-EE was used to construct the calibration standard curve as described in the Experimental Section. The insert illustrates the low concentration range area (0.005−5 ng/mL) of the same curve. The correlation coefficient for the triplicate curve was 0.999.

Table 2. Summary of the Calibration Standard Curve Validation in Female Rhesus Monkey Plasma

	intraday est. concn			interday validation summary			
nominal concn (ng/mL)	day 1	day 2	day 3	mean	SD	mean % CV	mean % accuracy
0.005 00	0.00600	0.00519	0.00499	0.00516	0.000387	7.5	103
	0.00553	0.00505	0.00500				
	0.00494	0.00500	0.00470				
0.0100	0.00952	0.0106	0.0100	0.00986	0.000433	4.4	99
	0.0101	0.00983	0.0100				
	0.00985	0.00984	0.00900				
0.0250	0.0261	0.0256	0.0240	0.0244	0.00177	7.2	98
	0.0221	0.0238	0.0240				
	0.0217	0.0252	0.0270				
0.0500	0.0480	0.0505	0.0510	0.0491	0.00213	4.3	98
	0.0491	0.0500	0.0500				
	0.0442	0.0507	0.0480				
0.100	0.0942	0.0990	0.0960	0.0973	0.00284	2.9	97
	0.0945	0.0994	0.102				
	0.0947	0.0959	0.100				
0.250	0.240	0.245	0.248	0.254	0.00895	3.5	101
	0.265	0.263	0.249				
	0.255	0.264	0.253				
0.500	0.495	0.497	0.519	0.508	0.0117	2.3	102
	0.514	0.491	0.505				
	0.519	0.513	0.522				
1.00	1.09	1.02	1.01	1.04	0.0584	5.6	104
	1.05	1.13	0.986				
	1.10	0.993	0.960				
5.00	5.40	4.74	4.80	5.06	0.281	5.6	101
	5.39	4.89	5.02				
	5.45	5.01	4.84				
20.0	20.0	19.2	19.4	19.2	0.367	1.9	96
	19.5	19.3	19.2				
	19.0	18.8	18.9				

[a] Three sets of triplicate female rhesus monkey plasma samples were spiked with 0.005 to 20 ng/mL ethinylestradiol in different days and analyzed to estimate the inter- and intra-day precision and accuracy and linearity of calibration standard curves as described in the Experimental Section. The correlation coefficient for day one, two, and three of the validation for each triplicate curve was 0.9988, 0.9998, and 0.9998, respectively.

extensive washes (10 washes/injection) of the syringe, valve, and injection loop, as described in the Experimental Section, the carryover was reduced to <0.1%, allowing us to reach the target LOQ of 5 pg/mL. The LOQ for the validated radioimmunoassays ranged from 8 to 20 pg/mL.[21] In the case of the validated GC/MS assay, an LOQ of 10 pg/mL was achieved.[21]

A linear calibration curve was constructed using the weighted ($1/X^2$) regression of the peak area ratio (analyte/internal standard area under curve) versus concentration of the triplicate calibration standards (Figure 4). The correlation was linear over a wide dynamic range from 0.005 to 20 ng/mL when deuterated EE was used as an internal standard. The correlation coefficient (R^2) obtained for each individual triplicate curve was in the range of 0.9988 to 0.9998. The mean interday accuracy ranged from 96 to 103% (<4% bias), and the mean interday precision for all standards was <7.5% (Table 2).

Table 3. Summary of the Quality Control Validation in Female Rhesus Monkey Plasma

		intraday est. concn			interday validation summary			
nominal concn (ng/mL)		day 1	day 2	day 3	mean	SD	% CV	% accuracy
0.0100		0.0100	0.00967	0.0099				
		0.0110	0.00985	0.0100	0.0100	0.000403	4.0	100
		0.0100	0.00972	0.0103				
	mean	0.0103	0.0097	0.0101				
	SD	0.000577	0.000093	0.000222				
	% CV	5.6	1.0	2.2				
	% accuracy	103	98	101				
0.0500		0.0480	0.0480	0.0535				
		0.0460	0.0490	0.0521	0.0507	0.00372	7.3	101
		0.0580	0.0530	0.0491				
	mean	0.0507	0.0500	0.0516				
	SD	0.00643	0.00265	0.00225				
	% CV	12.7	5.3	4.4				
	% accuracy	101	100	103				
0.500		0.520	0.509	0.519				
		0.509	0.490	0.503	0.508	0.0108	2.1	102
		0.500	0.522	0.500				
	mean	0.510	0.507	0.507				
	SD	0.0100	0.0161	0.0102				
	% CV	2.0	3.2	2.0				
	% accuracy	102	101	101				
5.00		4.91	5.21	4.91				
		5.00	5.14	4.98	5.02	0.101	2.0	100
		5.00	4.97	5.04				
	mean	4.97	5.11	4.98				
	SD	0.0537	0.125	0.0651				
	% CV	1.1	2.4	1.3				
	% accuracy	99	102	100				

[a] Three sets of triplicate female rhesus monkey plasma samples were spiked with 0.01, 0.05, 0.5, and 5 ng/mL ethinylestradiol on different days and analyzed to estimate the inter- and intraday precision and accuracy of the quality control samples as described in the Experimental Section.

Table 4. Individual and Mean Pharmacokinetic Parameters for Ethinylestradiol Following Intravenous or Oral Administration of EE to Four Female Rhesus Monkeys

pharmacokinetic parameter	monkey R061	monkey R001	monkey R007	monkey R013	mean	SD
AUC (i.v.)$_{(0-8\ h)}$[a], ng·h/mL	7.2	7.1	7.9	8.8	7.8	0.8
CLp[b], mL/min/kg	23	24	21	19	22	2.2
Vd_{ss}[c], L/kg	2.4	2.7	2.4	1.8	2.3	0.4
$t_{1/2}$[d], h	2.3	1.9	3.2	2.0	2.4	0.6
AUC (p.o.)[e], ng·h/mL	1.9	1.9	6.1	2.4	3.1	2.0
F[f]	0.009	0.009	0.026	0.009	0.013	0.009

[a] Intravenous (i.v.) AUC was calculated using linear trapezoidal approximation, including the estimated C_0. [b] Clearance (CLp) was estimated from the equation CLp = dose/$AUC_{(0-t)}$. [c] Volume of distribution at steady state (Vdss) was estimated from the equation Vdss = CLp × MRT. [d] Terminal half-life ($t_{1/2}$) was estimated using the 4-, 6-, and 8-h time points. [e] Oral (p.o.) AUC was calculated using linear trapezoidal approximation. [f] Bioavailability was calculated using the equation: F = AUC (p.o.) × dose (i.v.)/AUC (i.v.) × dose (p.o.).

The precision and accuracy values for all 3-day validated quality control replicates ($n = 36$) are summarized in Table 3. The intra- and interassay accuracy, determined at each QC level throughout the validated runs, remained below 3%. The mean intra- and interassay precision was <12.7 and 7.3% for all QC levels, respectively (Table 3). The percent deviation and coefficient of variation values reported for the validated radioimmunoassays were <10−20% and <7−18%, respectively.[21] Clearly, the current assay based on the MS/MS detection demonstrates superior accuracy (<3% bias) at all concentration ranges over the classical RIA.

Using our validated assay, the plasma concentration versus time profile of EE was determined in four female rhesus monkeys dosed intravenously (0.01 mg/kg) or orally (0.3 mg/kg)(Figure 4). The oral dose of EE selected in this study was one-third of the dose administered in the past pharmacokinetic investigation in the rhesus monkey, in which the samples were analyzed by the RIA (31). Plasma samples of only 50 μLwere used for our EE analysis, instead of the 0.5−1.0 mL used in RIA.[31] The analysis of all samples (130 samples), including triplicate standards and QCs, was completed in about 6 h, resulting in a 15-fold higher analysis throughput, as compared to the RIA method. The mean intravenous $AUC_{(0-8\ h)}$ was 7.8 ± 0.8 ng.hr/mL, which is consistent with a value published previously (9.3 ± 2.3 ng.hr/mL).[31] The estimated mean plasma clearance was 22 ± 2.2 mL/min/kg, also similar to a value published previously (17 ± 8 mL/min/kg).[31] The values for mean terminal half-life (2.4 ± 0.6 h), volume of distribution (2.3 ± 0.4 L/kg), and bioavailability (0.013 ± 0.009) were all within the ranges reported previously using a two-compartmental model.[31]

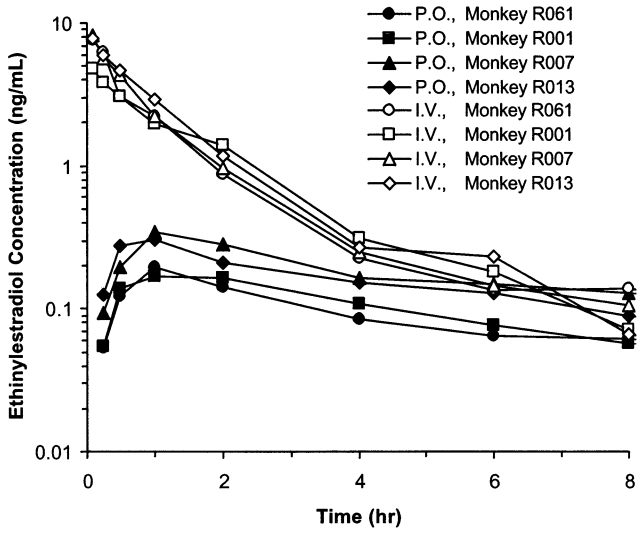

Figure 5. Plasma concentration versus time profile of ethinylestradiol in four female rhesus monkeys dosed intravenously or orally with EE. Four female rhesus monkeys were dosed orally (0.3 mg/kg) or intravenously (0.01 mg/kg) with EE, and the samples were analyzed as described in the Experimental Section.

CONCLUSIONS

The pharmacokinetic analysis of EE relies on a highly sensitive assay, capable of determining EE in plasma at low picogram-per-milliliter concentrations. In preclinical species, the restricted volumes of plasma, extensive metabolism of EE, and interferences from endogenous steroids, all add to the complexity of the trace analysis of EE. The rational derivatization of EE with dansyl chloride, to enhance electrospray ionization, contributed toward the development of a highly sensitive and specific method for rapid and accurate determination of EE in plasma from rhesus monkeys. The sensitivity, accuracy, and throughput of the LC/MS/MS assay were significantly higher than those of the RIA or GC/MS techniques. The assay required lower volumes of plasma, which allowed for the pharmacokinetic analysis to be conducted in individual animals, thereby eliminating errors due to the pooling of samples or interindividual variations. Currently, rats and other preclinical species are under investigation to determine the closest animal model to human for oral contraceptive interaction studies. The information from such studies would allow for the early screening of potential drug interaction and design of drugs that could be taken concomitantly with oral contraceptive preparations containing EE.

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