Published in final edited form as:

J Pharm Biomed Anal. 2012 December ; 71: 119–126. doi:10.1016/j.jpba.2012.08.022.

Pharmacokinetics of the estrogen receptor subtype-selective ligands, PPT and DPN: Quantification using UPLC-ES/MS/MS

Estatira Sepehr^a, Marketa Lebl-Rinnova^b, Meagan K. Mann^{b,1}, Samantha L. Pisani^c, Mona I. Churchwell^a, Donna L. Korol^{c,d}, John A. Katzenellenbogen^b, and Daniel R. Doerge^{a,*}

^aNational Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079, USA

^bDepartment of Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801

^cNeuroscience Program, University of Illinois at Urbana-Champaign, Urbana, IL 61801

^dDepartment of Psychology, University of Illinois at Urbana-Champaign, Champaign, IL 61820

Abstract

Estrogen receptor (ER) subtype specific agonists, diarylpropionitrile (DPN) for ER β and propylpyrazoletriol (PPT) for ER α , are pharmacological probes used frequently to define mechanisms for estrogen actions in vitro and in vivo. Quantitative analytical methodology was developed and validated for DPN and PPT, based on synthetic stable labeled analogs (DPN-*d*₄ and PPT-*d*₅) using isotope dilution liquid chromatographic tandem electrospray mass spectrometric detection. The validated method produced high sensitivity, with detection limits of 0.04–0.07 ng/ml serum. Serum pharmacokinetics were evaluated in Long-Evans rats following a single subcutaneous injection (2 mg/kg bw) of both compounds. The role of Phase II metabolism was evaluated using β -glucuronidase and arylsulfatase hydrolysis to measure total DPN and PPT in addition to the parent compounds. The pharmacokinetic properties of DPN and PPT reported could facilitate experimental designs requiring specified levels of receptor occupancy for quantitative comparisons of ER subtype specificities for natural and synthetic estrogens in vivo.

Keywords

estrogen receptor subtypes; pharmacokinetics; mass spectrometry

1. Introduction

Estrogen receptors (ER) transduce ligand binding into gene expression changes in target tissues through their actions as nuclear transcription factors and by regulation of extranuclear signaling pathways [1]. There are two ER subtypes, ER α and ER β , and discrimination between estrogenic effects elicited through one or the other of these receptors in tissues containing both has often relied on pharmacological agents with defined ligand specificity. The development of 2,3-bis(4-hydroxyphenyl)propionitrile (DPN) as ER β -specific [2] and 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1*H*-pyrazole (PPT) as ER α -specific

*Corresponding author: Daniel R. Doerge Daniel.Doerge@fda.hhs.gov Tel: +1 870 543 7943 Fax: +1 870 543 7720.

¹Current address: Department of Chemistry, Austin Peay State University, Clarksville, TN 37044

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

[3] agonists (Figure 1) has facilitated research to dissect pharmacologically the roles for ER α and ER β in vivo in a manner complementary to the use of selective ER-knockout mice [4,5].

While the potency of estrogen actions in tissues is related to ligand-receptor binding affinity, other cellular factors are important in determining overall potency, including receptor coactivator recruitment [6]. In addition, pharmacokinetic factors related to metabolism and disposition play a major role in determining the tissue levels that initiate estrogenic effects in vivo. The limited information available in rodents regarding serum levels of either DPN following subcutaneous injection and oral administration [7] or PPT following subcutaneous injection [4] is not adequate to evaluate pharmacokinetic behavior of these useful test compounds. The goals of the current investigation were to synthesize DPN and PPT labeled with stable isotopes as the basis for isotope dilution quantification of parent compounds (i.e., aglycones) and their Phase II metabolites and to determine serum pharmacokinetics in rats after single dose administration.

2. Materials and methods

2.1. Reagents

All solvents were HPLC grade and Milli-Q water was used throughout. Sigma-Chemical Co. (St. Louis, MO) supplied the glucuronidase/sulfatase (from *Helix pomatia*, type H-1) and sodium citrate. Fisher Scientific (Pittsburgh, PA) supplied methyl tert-butyl ether and acetonitrile. J.T. Baker (Austin, TX) supplied methanol.

2.2. Synthetic procedures

2.2.1 PPT—The synthesis of deuterated PPT consists of 5 steps, as depicted in Scheme 1. 1,3-Diketone **1**, prepared via a Claisen condensation, was alkylated with propargyl bromide, providing 2-propargyl-1,3-diketone **2**. Condensation with 4-methoxyphenyl hydrazine hydrochloride yielded propargyl pyrazole **3**, which was deuterated in presence of D₂ over a Pd catalyst. Several concurrent experiments showed the importance of the solvent chosen for the reaction. When neat methanol was used, the content of unlabeled material in intermediate **4** was more than 11% (ESI⁺ MS). Replacing methanol with toluene had a favorable effect on a content of deuterium in the intermediate. Finally, a mixture of CH₃OD with toluene-*d*₈ provided the best results (only about 1% of unlabeled compound). We also found that degassing the mixture before the catalyst was added and subsequent bubbling the mixture with D₂ significantly improved the efficiency of deuterium introduction. This procedure led to incorporation of five deuterium atoms into the propyl moiety. In the last step, methyl ethers were cleaved using BBr₃, and in this transformation neither exposure to this reagent nor an acidic workup had influence on the deuterium content of the final product (PPT-*d*₅).

1,3-Bis-(4-methoxy-phenyl)-propane-1,3-dione (1): To NaH (60% suspension in mineral oil, 240 mg, 6 mmol) was added DMSO (anhydrous, 12 ml) under N₂ at 0 °C. The mixture was then stirred at room temperature for 30 min and then cooled again so that during slow addition of methyl 4-methoxybenzoate (1 g, 6 mmol), under N₂, the temperature did not exceed 15 °C. Then the temperature was kept at 10 °C, and 4-methoxyacetophenone (540 mg, 3.6 mmol) was slowly added. Upon completion of the addition, the bath was removed, and the reaction was stirred at room temperature. The yellow mixture was poured onto ice (ca. 50 ml) containing phosphoric acid (85%, 250 μ l). The precipitate was filtered, washed with water and dried. The pale yellow product was recrystallized from hot EtOH to yield the product **1** as yellow needles (859 mg, 72%). MS (EI⁺): m/z 284.0, C₁₇H₁₆O₄ calculated

284.10. ^1H NMR (CDCl_3 , 500 MHz) δ 3.89 (s, 6H), 6.74 (s, 1H), 6.97–6.99 (d, 4H, $J = 9$ Hz), 7.95–7.97 (d, 4H, $J = 9$ Hz).

1,3-Bis-(4-methoxy-phenyl)-2-prop-2-ynyl-propane-1,3-dione (2): To a solution of **1** (3.24 g, 11.38 mmol) in DMSO (40 ml) and DCM (20 ml) with anhydrous K_2CO_3 (6.57 g, 47.5 mmol), cooled to 0°C , was added propargyl bromide (80% solution in toluene, 1.33 ml, 12.51 mmol). The mixture was stirred at 0°C for 2 h and then at room temperature for 18 h. The reaction mixture was filtered, the K_2CO_3 was washed with DCM, and the collected solution was washed with water, dried over MgSO_4 and concentrated. The crude material **2** was purified on silica gel (30% EtOAc/Hexanes) to yield 2.96 g (80%) of the product as oil. HRMS (FAB^+): m/z 323.12830 [$\text{M}+\text{H}$], $\text{C}_{20}\text{H}_{18}\text{O}_4+\text{H}$ calculated 323.12834. ^1H NMR (CDCl_3 , 500 MHz) δ 1.56 (s, 1H), 2.98–3.00 (dd, 2H, $J = 7.1, 2.5$ Hz), 3.86 (s, 6H), 5.33–5.36 (t, 1H, $J = 7$ Hz), 6.91–6.93 (d, 4H, $J = 9$ Hz), 7.97–7.99 (d, 4H, $J = 9$ Hz).

1,3,5-Tris-(4-methoxy-phenyl)-4-prop-2-ynyl-1H-pyrazole (3): To a solution of **2** (1.28 g, 3.96 mmol) in DMF (15 ml) was added 4-methoxyphenyl hydrazine hydrochloride (2.07 g, 11.88 mmol) and conc. HCl (395 μl , 5.92 mmol), and the reaction was stirred at 110°C overnight. The reaction mixture was diluted with EtOAc, washed with water and brine, dried over MgSO_4 and concentrated. The crude material was purified on silica gel (20–30% EtOAc/Hexanes) to yield 1.09 g (65%) of the pure product **3** as oil. HRMS (FAB^+): m/z 425.1865 [$\text{M}+\text{H}$], $\text{C}_{27}\text{H}_{24}\text{N}_2\text{O}_3+\text{H}$ calculated 425.1865. ^1H NMR (CDCl_3 , 500 MHz) δ 3.01–3.02 (t, 1H, $J = 2.79$ Hz), 3.32 (d, 2H, $J = 2.57$ Hz), 3.75 (s, 3H), 3.78 (s, 3H), 3.81 (s, 3H), 6.91–6.92 (d, 2H, $J = 9$ Hz), 6.99–7.01 (d, 2H, $J = 9$ Hz), 7.06–7.07 (d, 2H, $J = 8.79$ Hz), 7.19–7.20 (d, 2, $J = 9$ Hz), 7.22–7.23 (d, 2H, $J = 9$ Hz), 7.77–7.79 (d, 2H, $J = 9$ Hz).

1,3,5-Tris-(4-methoxy-phenyl)-4-($\beta,\beta,\gamma,\gamma\text{-}^2\text{H}_5$)propyl-1H-pyrazole (4): To an argon-flushed mixture of Pd on charcoal (10%, 150 mg) in CH_3OD (5 ml) was added a solution of pyrazole **3** (215 mg, 0.5 mmol) in toluene- d_8 (5 ml) that was also flushed with argon. The mixture was repeatedly flushed with argon and evacuated. After the final evacuation, an argon inlet was replaced with D_2 inlet, and the reaction mixture was flushed with deuterium gas for 20 min and then stirred under a balloon filled with D_2 for 24 h. The mixture was filtered through Celite that was washed with CH_3OD . The filtrate was concentrated and purified on silica gel (20–30% acetone/hexanes) to yield an oil that was triturated with MeOH to yield the product **4** (166 mg, 77%) as an off-white solid. HRMS (FAB^+): m/z 433.24910 [$4\text{D}+1$], $\text{C}_{27}\text{H}_{24}\text{D}_4\text{N}_2\text{O}_3+\text{H}$ calculated 433.24913. ^1H NMR (CDCl_3 , 500 MHz) δ 0.74–0.78 (m, 0.83 H), 1.38 (m, 0.54 H), 2.56 (bs, 1.56 H), 2.58 (bs, 0.36 H), 3.78 (s, 3H), 3.84 (s, 3H), 3.87 (s, 3H), 6.78–6.80 (d, 2H, $J = 8.79$ Hz), 6.89–6.90 (d, 2H, $J = 8.79$ Hz), 6.98–7.00 (d, 2H, $J = 8.79$ Hz), 7.14–7.16 (d, 2H, $J = 8.79$ Hz), 7.20–7.22 (d, 2H, $J = 9.22$ Hz), 7.70–7.72 (d, 2H, $J = 8.79$ Hz).

1,3,5-Tris-(4-hydroxy-phenyl)-4-($\beta,\beta,\gamma,\gamma\text{-}^2\text{H}_5$)propyl-1H-pyrazole (PPT- d_5): Pyrazole **4** (164 mg, 0.379 mmol) was dissolved in dry DCM (4 ml) and cooled under N_2 to -76°C . Then BBr_3 (1 M solution on DCM, 2.27 mmol) was added dropwise, and the mixture was slowly warmed up to room temperature and stirred overnight. The reaction was quenched with water (2 ml) and stirred with EtOAc (30 ml) for 20 min. Then the mixture was washed with 10% citric acid, water and brine in a separatory funnel. The organic phase was dried over MgSO_4 and concentrated. The crude material was purified on silica gel (50% acetone/hexanes) to give an off-white solid that was re-crystallized from MeOH/DCM to yield the product as a white solid (115 mg, 78%). HRMS (ESI^+): m/z 392.2007 [$\text{M}+\text{H}$], $\text{C}_{24}\text{H}_{18}\text{D}_5\text{N}_2\text{O}_3+\text{H}$ calculated 392.2023. ^1H NMR ($\text{DMSO}-d_6$, 500 MHz) δ 0.66–0.70 (m, 0.83 H), 1.26–1.31 (m, 0.62 H), 2.45 (bs, 1.56 H), 2.48 (bs, 0.36 H), 6.65–6.67 (d, 2H, $J =$

8.79 Hz), 6.75–6.77 (d, 2H, $J = 8.58$ Hz), 6.82–6.84 (d, 2H, $J = 8.79$ Hz), 6.99–7.01 (dd, 4H, $J = 8.68$ Hz, 2.04 Hz), 7.47–7.49 (d, 2H, $J = 8.79$ Hz), 9.51 (s, 1H), 9.57 (s, 1H), 9.66 (s, 1H).

2.2.2 DPN—The synthesis of DPN- d_4 consists of 6 steps, as depicted in Scheme 2. The first deuterium was introduced early in the synthesis by a deuterodeiodination reaction using Pd catalyst and deuterium gas. Deuterated benzoic acid methyl ester **5** was reduced with LiAlD_4 to alcohol **6**, which was further oxidized to deutoaldehyde **7**. Condensation with 4-methoxyphenylacetonitrile gave cyanostilbene **8**. Up to this point, no loss of deuterium was detected either by ^1H NMR or MS. However, reduction of acrylonitrile **8** using NaBD_4 in DMF to give the intermediate **9**, proved to be a bottleneck of the whole synthesis. Maximum yields of this reaction did not exceeded 40%, and ^1H NMR revealed loss of about 30% deuterium in the position α to the nitrile. Interestingly, the ^1H NMR of crude material displayed only traces of this isotopic exchange, but after re-crystallization from EtOH a sharp singlet appeared at δ 3.90. Treatment with BBr_3 then provided the final deuterated DPN (DPN- d_4). Additional deuterium-protium exchange in position α occurs during acid work-up (10% aq. citric acid), which is necessary after BBr_3 treatment. The loss of deuterium in this position in the final compound is 53%.

4-Methoxy-(3- $^2\text{H}_1$)benzoic acid methyl ester (5): 3-Iodo-4-methoxy-benzoic acid methyl ester (3 g, 10.26 mmol) was dissolved in MeOH (150 ml) with TEA (14.3 ml, 102.75 mmol), and Pd (10% on charcoal, 1.08 g, 1.02 mmol) was added under N_2 . The flask was evacuated, and a D_2 filled balloon was attached. When the balloon content was consumed another balloon was attached. The reaction was stirred for 30 h at room temperature. The mixture was filtered over Celite under N_2 , and the filtrate was concentrated under reduced pressure. The residue was treated with hexane/EtOAc (9:1), and a white precipitated salt was removed by filtration. The filtrate was concentrated, and the crude material was purified on silica gel (DCM) to yield the product as a white solid **5** (1.4 g, 83%). ^1H NMR (CDCl_3 , 500 MHz) δ 3.86 (s, 3H), 3.88 (s, 3H), 6.91–6.92 (m, 1H), 7.98–8.00 (m, 2H).

(4-Methoxy-(3- ^2H)phenyl)-($^2\text{H}_2$)methanol (6): Ester **5** (1.9 g, 11.4 mmol) was dissolved in dry diethyl ether (20 ml) and added under N_2 , slowly over 30 min, to a solution of LiAlD_4 (1 g, 24 mmol) in dry diethyl ether. The reaction mixture was stirred for 3.5 h at rt, then ice (app. 20 ml) was added, and HCl (1M aq. solution, app. 30 ml) was used to acidify the mixture. The product was extracted with EtOAc, washed with water and brine, dried over MgSO_4 and concentrated. The crude material was purified over silica gel (50% EtOAc/hexanes) to yield the product **6** as a colorless oil (1.59 g, 98%). HRMS (EI^+): m/z 141.0868 [M^+], $\text{C}_8\text{H}_7\text{D}_3\text{O}_2$ calculated 141.0869. ^1H NMR ($\text{DMSO}-d_6$, 500 MHz) δ 3.73 (s, 3H), 5.00 (s, 1H), 6.86–6.88 (m, 1H), 7.21–7.23 (m, 2H).

4-Methoxy-(3- ^2H)benzaldehyde-(1- ^2H)(7): To a solution of alcohol **6** (200 mg, 1.42 mmol) in DCM (20 ml) with 2,6-lutidine (181 μl , 1.55 mmol) at 0°C was added a solution of Dess-Martin periodinane (1.08 g, 2.56 mmol) in DCM (6.7 ml). The mixture was stirred for 1.5 h at 0°C and quenched with $\text{Na}_2\text{S}_2\text{O}_3$ (10 % aq. solution, 50 ml) and sat. NaHCO_3 (50 ml). The aqueous layer was extracted with DCM, and the combined organic extract was washed with water and brine, dried over MgSO_4 and concentrated to yield an oil that was purified on silica gel (20% EtOAc/hexanes) to give 168 mg (86%) of the product **7** as oil. ^1H NMR ($\text{DMSO}-d_6$, 500 MHz) δ 3.87 (s, 3H), 7.13–7.15 (m, 1H), 7.87–7.89 (m, 2H).

3-(4-Methoxy-(3- ^2H) phenyl)-2-(4-methoxy-phenyl)-(3- ^2H)acrylonitrile (8): To a solution of (4-methoxy-phenyl)-acetonitrile (841 μl , 6.19 mmol) and aldehyde **7** (777 mg, 5.63 mmol) in abs. EtOH (2.2 ml) was added a solution of KOH (40% aq. sol., 1.42 ml)

diluted with EtOH (2.84 ml) at room temperature. The reaction mixture was stirred at room temperature until a dense precipitate formed, and then it was left at room temperature overnight. The precipitate was collected by filtration and then washed with water and 30% EtOH/water solution. Following drying, the product **8** was obtained as pale yellow needles (1.15 g, 77%). HRMS (EI⁺): *m/z* 267.1232 [M⁺], C₁₇H₁₃D₂NO₂ calculated 267.1228. ¹H NMR (CDCl₃, 500 MHz) δ 3.86 (s, 3H), 3.88 (s, 3H), 6.95–6.99 (m, 3H), 7.58–7.60 (dt, 2H, *J* = 2.2 Hz, 9.03 Hz), 7.85–7.88 (m, 2H).

3-(4-Methoxy-(3-²H) phenyl)-2-(4-methoxy-phenyl)-(2,3,3-²H₃)propionitrile (9): A solution of NaBD₄ (78 mg, 1.86 mmol) in dry DMF (2.5 ml) was added to a solution of nitrile **8** (247 mg, 0.93 mmol) in dry DMF (1.5 ml) at room temperature. The reaction mixture was stirred at 60 °C for 8 h and then at 40 °C overnight. The reaction was cooled slowly to room temperature and then to 0 °C in an ice bath, and D₂O (10 ml) was added slowly. The suspension was neutralized with DCl (35% in D₂O) dropwise. The product was extracted with diethylether, washed with D₂O and brine, dried over MgSO₄ and concentrated. The product was crystallized from EtOH to yield white solid (103 mg, 40%). HRMS (EI⁺): *m/z* 271.1510 [M⁺], C₁₇H₁₃D₄NO₂ calculated 271.1510. ¹H NMR (CDCl₃, 500 MHz) δ 3.80 (s, 3H), 3.82 (s, 3H), 3.91 (s, 0.3 H), 6.82–6.84 (d, 1H, *J* = 9.03 Hz), 6.87–6.89 (dt, 2H, *J* = 8.79 Hz, 2.2 Hz), 7.03–7.05 (m, 2H), 7.15–7.16 (dt, 2H, *J* = 8.79 Hz, 2.2 Hz).

3-(4-Hydroxy-(3-²H) phenyl)-2-(4-hydroxy-phenyl)-(2,3,3-²H₃)propionitrile (DPN-d₄): Nitrile **9** (100 mg, 0.37 mg) in dry DCM (2 ml) was cooled to –77 °C under N₂, BBr₃ (1 M in DCM, 1.48 ml, 1.48 mmol) was added slowly and the mixture was stirred and slowly warmed up to room temperature overnight. The reaction was quenched with water (2 ml) and partitioned between 10% aq. citric acid and EtOAc. The organic phase was washed with brine, dried over MgSO₄ and concentrated. The crude material was dissolved in acetone (3 ml), diluted with dichloromethane (27 ml) and passed through a short silica gel column. The filtrate was concentrated, and the product was crystallized from EtOH/dichloromethane to yield the white solid product (80.5 mg, 89%). HRMS (EI⁺): *m/z* 243.11939 [M⁺], C₁₅H₉D₄NO₂ calculated 243.11973. ¹H NMR (CDCl₃, 500 MHz) δ 4.25 (s, 0.5 H), 6.65–6.67 (d, 1H, *J* = 8.79 Hz), 6.73–6.75 (dt, 2H, *J* = 8.79 Hz, 2.2 Hz), 7.00–7.02 (m, 2H), 7.13–7.15 (dt, 2H, *J* = 8.55 Hz, 1.95 Hz), 9.28 (s, 1H), 9.52 (s, 1H).

2.3. Liquid chromatography and mass spectrometry

2.3.1. Ultra-performance liquid chromatography (UPLC)—UPLC separation was performed using a Waters Acquity Liquid handling system from Waters Corp. (Milford, MA). Chromatography was performed using a UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm, Waters), protected with guard column packed with C18 materials (VanGuard BEH C18, 1.7 μm, Waters) with gradient elution of water and acetonitrile. Initial gradient conditions were set at 35% ACN and held for 2.8 min before incorporating a linear gradient increasing to 55% ACN for 0.1 min. At 2.9 min, the gradient was programmed to initial conditions to re-equilibrate the column for 0.6 min (Total run time 3.5 min). The flow rate for all analyses was 0.4 ml/min. All separations of standards and serum samples were performed at room temperature. All sample extracts were maintained in the autosampler at 20 °C while awaiting injection. All injection volumes were 15 μl.

2.3.2. Mass spectrometry—The entire effluent from UPLC column was directed into the electrospray (ES) source of a Waters Quattro Micro triple quadrupole mass spectrometer (Waters Corp., Milford, MA). For DPN and PPT analysis, two time functions were utilized: first time function (0.0–2.05 min), monitored the [M–H][–] ions for labeled and unlabeled DPN (*m/z* 242 → 132 and 238 → 132, respectively) using a dwell time of 0.08 s and inter-

channel delay time of 0.005 s in multiple reaction monitoring mode (MRM) for ES/MS, at cone voltage of 25 V. In the second time function, (2.05–3.50 min) positive ions $[M+H]^+$ were monitored for labeled and unlabeled PPT (m/z 392→225 and 387→225, respectively) using dwell time of 0.08 s and inter-channel delay time of 0.005 s in MRM mode at cone voltage of 50 V. Additional MRM transitions were acquired for PPT (m/z 387→120) and DPN (m/z 238→211), to serve as confirmation in case of coeluting interferences. Mass spectrometer operating conditions included: capillary voltage, 3.0 kV; extractor voltage, 3.0 V, RF lens voltage, 0.3 V; source temperature, 100 °C; and desolvation gas flow, 750 L/h. For all MS/MS measurements, Argon (Ar) gas cell pressure 4.3×10^{-3} mbar and collision energy of 10 and 37 eV were used for DPN and PPT, respectively.

2.3.2.1. Isotope dilution: Isotopically labeled DPN and PPT were characterized using LC-UV and full scan LC-ES/MS. The concentration of labeled compounds was determined using LC-UV ($\lambda = 254$ nm, and $\lambda = 227$ nm for PPT and DPN, respectively), and the responses from known amounts of the corresponding unlabeled compounds were used as external standards. The DPN electrospray mass spectrum for the deprotonated molecule showed isotopic content of: d_4 (m/z 242): 50%; d_3 (m/z 241): 49%; d_2 (m/z 240): 1%; d_0 (m/z 238): <0.03%. The PPT electrospray mass spectrum for the protonated molecule showed isotopic content of: d_5 (m/z 392), 44 %; d_4 (m/z 391), 35 %; d_3 (m/z 390), 16%; d_2 (m/z 389), 5 %; d_0 (m/z 387), 0.08%.

A linear calibration curve was constructed from mixtures of each labeled PPT and DPN at a constant concentration (10 pg/injection for PPT and 30 pg/injection for DPN) along with the respective unlabeled PPT and DPN at concentrations from one tenth of the labeled concentration up to fifty-fold higher (1–500 pg/injection for PPT and 3–1500 pg/injection for DPN). The calibration curve had to have a coefficient of determination (r^2) of 0.996 or better. For each calibration curve, peak area ratios of the analyte to the internal standard (response factor) were plotted against the known analyte concentration. The resulting slope and y-intercept values for each analyte were used to determine analyte values of unknown samples using the best-fit line equation ($y = mx + b$). The response factors for each set of labeled/unlabeled compounds (2.42 for DPN and 1.60 for PPT) were checked daily by analyzing mixtures of labeled and unlabeled standards.

2.4. Serum preparation and purification

Rat serum samples were analyzed for both total and aglycone PPT and DPN using the following method. On the day of the analysis, rat serum samples were thawed at room temperature. Depending on the range of PPT and DPN concentrations, the amounts of serum analyzed were typically 10 μ l for analysis of total serum concentrations and 100 μ l for aglycone concentrations (see below). The serum samples were mixed with 340 μ l of sodium citrate buffer (25 mM, pH 5.0) and 50 μ l of the internal standard solution (2 ng/ml PPT and 6 ng/ml DPN in methanol/water 50/50). Aliquots of 400 μ l were applied to supported liquid extraction plates (SLE, Biotage, Charlotte, NC) and allowed 5 min for complete absorption of the sample. Then the plate was eluted with two 900 μ l aliquots of methyl *t*-butylether. The extracts were evaporated to dryness using a heated concentrator SEPDry 96 (Biotage) under a nitrogen stream (Flow rate: upper 60 l/min, lower 40 l/min; temperature: upper 40 °C, lower 60 °C). The residues were reconstituted in 60 μ l of methanol, followed by 60 μ l milli-Q water. A 15 μ l aliquot of the extracted sample was injected onto the analytical column.

A kinetic experiment was conducted (0, 1, 2, 4, 16, 24, and 48 h time points) to determine the minimum incubation time required for maximal enzyme hydrolysis for PPT and DPN conjugates in pooled serum samples collected from dosed rats. The concentrations of total

(i.e., aglycone + glucuronidase/sulfate conjugates) PPT and DPN were monitored by UPLC/MS/MS. Maximal deconjugation of PPT and DPN was detected at 4 h and was stable thereafter out to 48 h (data not shown). For determination of total serum PPT and DPN, serum samples were added to 240 μ l of citrate buffer and 50 μ l of the internal standard solution, incubated with 100 μ l of *H. pomatia* glucuronidase/sulfatase mixture (2 units Sulfatase H-1, Sigma Chemical Co., Lt. Louis, MO, containing 300 units/mg β -glucuronidase and 10 units/mg arylsulfatase) at 37 °C for 4 h, and then prepared as described above. Aglycone concentrations of PPT and DPN were quantified without using the enzymatic hydrolysis step.

2.5. Quality control

Quality control (QC) samples were prepared by spiking control rat serum at four concentration levels [0 ng/ml (QC negative, using charcoal stripped Sprague-Dawley rat serum), 0.5 ng/ml (lower limit of quantification), 5 ng/ml (QC low), 25 ng/ml (QC medium), and 100 ng/ml (QC high)]. In each sample set, two to three levels of the quality control samples, blank rat serum, and a mixture of labeled and unlabeled standards were included. Representative chromatograms are presented (Fig. 2) for blank rat serum, blank serum spiked with PPT and DPN, and serum from a dosed rat (dosing and blood collection described below).

2.6. Animal handling and dosing

Three-month-old virgin Long-Evans female rats were obtained from Harlan (Barrier 202, Indianapolis, IN). Rats were individually housed in plastic cages on a 12:12 hour light:dark cycle, with *ad libitum* access to food and water. Following at least two days of acclimation to the vivarium, rats were bilaterally ovariectomized. Rats were ovariectomized after anesthesia with isoflurane and pre-operative injections of penicillin (100,000 units/kg, *i.m.*) and the analgesic carprofen (5 mg/kg, *s.c.*). Drinking water was supplemented with ibuprofen (Children's Motrin™, 2.35 ml/ 500 ml) for post-surgical pain management. From the day of surgery, rats were maintained on phytoestrogen-free AIN-93G chow (Research Diets, New Brunswick, NJ). All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and were in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Dosing solutions of DPN and PPT were prepared by finely pulverizing and homogenously suspending the solids in sesame oil at a concentration of 2.2 mg/ml. The volumes administered were determined from individually measured body weights using the relationship 0.91 μ l/g body weight (bw).

Two months after ovariectomy, three rats each received consecutive subcutaneous injections, one of DPN and another of PPT (2 mg/kg bw each) in sesame oil, and blood samples taken 15 min, 30 min, 1 h, 2 h, 4 h, 8 h and 24 h later. Blood samples were drawn by using a scalpel blade to make a small (< 3 mm) nick at the distal tail, and droplets were gently milked into glass test tubes to obtain a volume of approximately 100 μ L. Twenty-four hours following treatment, rats were overdosed with sodium pentobarbital (75 mg/kg bw), decapitated, and trunk blood collected. All blood samples were allowed to clot at room temperature then centrifuged at 2100 x g for 15 min at room temperature, and the serum was collected and frozen at -80 °C until analyzed.

2.7. Pharmacokinetic analysis

Plots of serial serum concentrations of total and aglycone DPN or PPT vs. time following subcutaneously administration to individual rats were analyzed using model-independent pharmacokinetic analysis (PK Solutions 2.0 software, Summit Research Services, Montrose,

CO). Natural log-linear plots were fit to up to three kinetic phases corresponding to elimination, distribution, and absorption. The first-order elimination rate constants (k_{elim}) were determined from the terminal slope of the respective curve. The first-order distribution and rate constants were determined after subtracting the contribution from the terminal elimination phase of the respective curve (i.e., feathering). Half-lives were determined from rate constants using the relationship: half-life ($t_{1/2}$) = $\ln 2/k$. Internal exposures to DPN or PPT were determined either as AUC, determined by using the trapezoidal rule, or as C_{max} , which was determined by graphical inspection. All data points collected were used for the graphical analyses described.

2.8. Statistical analysis

Pharmacokinetic parameters determined from plots of individual adult rat data were analyzed statistically as mean $\pm$ standard deviation.

3. Results

3.1. Method validation

3.1.1. Detection limit—The limit of detection (LOD) for PPT and DPN was estimated by spiking blank rat serum samples with known amounts of each compounds and a constant amount of internal standard, using the lowest concentration of the standards and defining the LOD as signal-to-noise ratio (S/N) of 3. The limits of detection for PPT and DPN from analysis of 100 μ L of serum were 0.04 and 0.07 ng/ml, respectively.

3.1.2. Accuracy—To demonstrate the accuracy of the method, control rat serum containing undetectable amounts of PPT and DPN was spiked with PPT and DPN at two different concentrations (0.5 and 5 ng/ml) for aglycones and (5 and 25 ng/ml) for totals. Serum samples were analyzed in quadruplicate and subjected to the complete extraction and analytical processes on two separate days. For each serum sample, the measured value was compared to the calculated value. The accuracies were 89–112% of the concentration added (Table 1).

3.1.3. Precision—The precision of the method was determined by preparing and assaying four replicates each of two different concentrations of PPT and DPN. Table 1 shows the single laboratory relative standard deviation (RSD) values range between 0.1–6.5%. The criteria for acceptability of the data included accuracy within $\pm 15\%$ SD from the nominal values and a precision of within $\pm 15\%$ RSD [8].

3.1.4. System stability—The stability of the system was monitored by injecting the known concentration of the internal standard (IS) throughout a sample set run. The peak areas of IS obtained at the beginning of the sample set were used as the reference to determine the system stability at subsequent points. The system was determined to be stable because assay values were within acceptable limits of accuracy (i.e. $\pm 15\%$ SD) and precision (i.e. $\pm 15\%$ RSD).

3.1.5. Recovery of PPT and DPN from rat serum—Recoveries were determined by spiking the labeled and unlabeled standards into 100 μ L of control rat serum and taking them through the entire extraction and analytical procedures. A minimum of four replicate measurements were performed on different days. Recoveries were determined on two separate days by comparing the concentrations for the PPT and DPN in the extracts of spiked samples with those obtained from fortification of blank serum extracts with standards just before analysis. Recoveries were 84–88% for DPN and 85–97% for PPT. The degree of signal suppression by matrix components were determined by comparing responses for

fortified serum extracts vs. neat standards in quadruplicate on two different days to be 3–14% for DPN and 26–32% for PPT. Also, an injection of the blank matrix was made to determine the matrix background (Figure 2).

3.2. Pharmacokinetics of DPN and PPT

Serum pharmacokinetics of DPN and PPT were determined in ovariectomized female Long-Evans rats as part of a larger investigation of ER-specificity in determining cognitive responses to botanical estrogens [9,10]. As opposed to estrogens consumed orally for therapeutic reasons, DPN and PPT are used primarily as diagnostic probes for ER-subtype specificity *in vitro* and *in vivo*, and are typically administered by injection. For this reason, the pharmacokinetics were investigated using the subcutaneous route of administration. In order to minimize the use of animals, simultaneous dosing with 2 mg/kg bw of each compound was used. Because of the molecular weight difference, the doses expressed on a molar basis were 8.4 $\mu\text{mol/kg}$ bw for DPN and 5.2 $\mu\text{mol/kg}$ bw for PPT. Total and aglycone DPN levels were maximal in serum at the 0.5 h time point (Figure 3), but maximal PPT levels were observed at 1 h (Figure 4). The mean C_{max} values for DPN and PPT were 1.0 and 0.35 μM , (Table 2). Using an assumption of dose linearity, this represents 0.1 μM per $\mu\text{mol/kg}$ bw and 0.07 μM per $\mu\text{mol/kg}$ bw administered for DPN and PPT, respectively. Both DPN and PPT displayed short distribution half-times, consistent with rapid transfer to tissues, and had similar elimination half-times (Table 2). The fraction of C_{max} comprised of aglycone was 36% for DPN and 75% for PPT. For the other measure of internal exposure, $\text{AUC}_{0-\infty}$ (Table 3), the dose-corrected values were 0.3 $\mu\text{mol} \times \text{h/l} \times \text{kg}$ bw and 0.4 $\text{h/l} \times \text{kg}$ bw for aglycone DPN and PPT, respectively. The fraction of $\text{AUC}_{0-\infty}$ comprised of aglycone was 13% for DPN and 69% for PPT.

4. Discussion

While ER subtype specificity and ligand affinity are important determinants of physiological effects *in vivo*, metabolic and pharmacokinetic factors also affect estrogenic potencies and activities. Like many endocrine-active compounds containing multiple phenolic moieties, DPN and PPT are subject to Phase II conjugation reactions. Based on the fraction of each compound as the unconjugated and therefore active form, in either the maximal level (C_{max}) or the time-weighted level ($\text{AUC}_{0-\infty}$), DPN is more extensively converted to conjugates than PPT (36 and 13% vs. 75 and 69%, respectively) following subcutaneous administration. As a result, the maximal level of DPN aglycone exceeded that of PPT aglycone by 1.7-fold, even though the dose-corrected AUC for PPT was higher. It is not clear from these data whether differences in C_{max} are solely due to pharmacokinetic differences or whether differences in rates of absorption from the site of injection due to solubility differences are also contributing. The apparent pharmacokinetic differences could conceivably influence responses for either fast estrogen signaling through extra-nuclear pathways or the classical nuclear transcription pathway [1,11]. The greater susceptibility of DPN to conjugation by systemic Phase II metabolism suggests that oral bioavailability of DPN could be lower than that for PPT because additional Phase II metabolism in the GI tract is encountered after oral administration [12].

Receptor binding affinities for ERs have been reported for DPN [2] and PPT [3], which can be compared with circulating levels in rats to estimate the degree of receptor activation in tissues for a specified dosing regimen (Figures 3 and 4). For DPN, the binding affinities (i.e., K_d) for ER α and ER β are approximately 400 nM and 5 nM, respectively. Similarly for PPT, the respective binding affinities are 2 nM and 1000 nM. Following subcutaneous injection of 2 mg/kg bw, the degree of receptor occupancy for ER β by DPN is predicted to be high for an extended time period, whereas ER α occupancy would be below 50% at all time points. Similarly for the same dosing with PPT, ER α occupancy would be high for an

extended time period while ER β occupancy would be below 50% at all time points. While tissue levels for lipophilic compounds like DPN and PPT are often higher than blood concentrations, the pharmacokinetic data presented here provide a framework for systematic evaluation of estrogenic effects mediated by ER subtypes based on both ligand binding specificity and internal dosimetry.

5. Conclusions

The pharmacokinetic information reported here extend previous reports and can facilitate future study designs assessing ER subtype-specificity *in vivo*, based on predictions of internal exposures to DPN and PPT, relative to static concentrations used *in vitro*. In this way, ER occupancy profiles can be better correlated with the pharmacological activity of these model agonists.

Acknowledgments

The authors acknowledge funding from Grant Number P50AT006268 to J.A.K. and D.R.D. from NIH, NIA, NCCAM, ODS, and NCI, as well as R37DK015556 to J.A.K. and NSF IOB 0520876 and IOS 1118414 to D.L.K. ES acknowledges support of a fellowship from the Oak Ridge Institute for Science and Education, administered through an interagency agreement between the U.S. Department of Energy and the U.S. Food and Drug Administration. The authors have no conflicts of interest to report. The views presented in this article do not necessarily reflect those of the U.S. Food and Drug Administration.

7. References

- [1]. Hammes SR, Levin ER. Extranuclear steroid hormone receptors: Nature and actions. *Endocrinol. Rev.* 2007; 28:726–741.
- [2]. Meyers MJ, Sun J, Carlson KE, Marriner GA, Katzenellenbogen BS, Katzenellenbogen JA. Estrogen receptor-beta potency-selective ligands: structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. *J. Med. Chem.* 2001; 44:4230–4251. [PubMed: 11708925]
- [3]. Stauffer SR, Coletta CJ, Tedesco R, Nishiguchi G, Carlson K, Sun J, Katzenellenbogen BS, Katzenellenbogen JA. Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-alpha-selective agonists. *J. Med. Chem.* 2000; 43:4934–4947. [PubMed: 11150164]
- [4]. Harris HA, Katzenellenbogen JA, Katzenellenbogen BS. Characterization of the biological roles of the estrogen receptors, ER α and ER β , in estrogen target tissues *in vivo* through the use of an ER α -selective ligand. *Endocrinology.* 2002; 143:4172–4177. [PubMed: 12399409]
- [5]. Frasor J, Barnett DH, Danes JM, Hess R, Parlow AF, Katzenellenbogen BS. Response-specific and ligand dose-dependent modulation of estrogen receptor (ER) alpha activity by ERbeta in the uterus. *Endocrinology.* 2003; 144:3159–3166. [PubMed: 12810572]
- [6]. Jeyakumar M, Carlson KE, Gunther JR, Katzenellenbogen JA. Exploration of dimensions of estrogenic potency. Parsing ligand binding and coactivator binding affinities. *J. Biol. Chem.* 2011; 286:12971–12982. [PubMed: 21321128]
- [7]. Patisaul HB, Burke KT, Hinkle RE, Adewale HB, Shea D. Systemic administration of diarylpropionitrile (DPN) or phytoestrogens does not affect anxiety-related behaviors in gonadally intact male rats. *Horm. Behav.* 2009; 55:3319–328.
- [8]. U.S. Food and Drug Administration. Guidance for industry, bioanalytical method validation, US Department of Health and Human Services Food and Drug Administration, Center for Drug Evaluation and Research (CDER). 2001. 2001 (<http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf>)
- [9]. Neese SL, Korol DL, Katzenellenbogen JA, Schantz SL. Impact of estrogen receptor alpha and beta agonists on delayed alternation in middle-aged rats. *Horm. Behav.* 2010; 58:878–890. [PubMed: 20816967]

- [10]. Pisani SL, Neese SL, Doerge DR, Helferich WG, Schantz SL, Korol DL. Acute genistein treatment enhances place learning and impairs response learning in young adult female rats. *Horm. Behav.* unpublished.
- [11]. Le HH, Belcher SM. Rapid signaling actions of environmental estrogens in developing granule cell neurons are mediated by estrogen receptor β . *Endocrinology*. 2010; 151:5689–5699. [PubMed: 20926581]
- [12]. Tukey RH, Strassburg CP. Genetic multiplicity of the human UDP-glucuronosyltransferases and regulation in the gastrointestinal tract. *Mol. Pharmacol.* 2001; 59:405–411. [PubMed: 11179432]

Highlights

UPLC/MS/MS was used to quantify estrogen receptor α and β subtype-specific ligands PPT and DPN in rat serum

The impact of Phase II metabolism on internal exposures to DPN and PPT was evaluated

Combining PK and ligand binding properties of DPN and PPT permit targeting levels of receptor occupancy in vivo

Propylpyrazoletriol (PPT)
mw 386

Diarylpropionitrile (DPN)
mw 239

Figure 1.
Structures of DPN and PPT.

Figure 2.

Representative multiple reaction monitoring chromatograms of PPT and DPN in rat serum: (A) Control rat serum containing labeled internal standards; (B) control rat serum spiked with unlabeled PPT and DPN at (25 ng/ml) and IS; and (C) rat serum sample obtained 0.5 hr after subcutaneous injection of PPT and DPN (2 mg/kg bw each).

Figure 3. Serum time-concentration profiles for total and aglycone DPN in rat serum following a single subcutaneously injected dose (2 mg/kg bw).

Figure 4. Serum time-concentration profiles for total and aglycone PPT in rat serum following a single subcutaneously injected dose (2 mg/kg bw).

Scheme 1.
Synthesis of PPT- d_5 .

Scheme 2.
Synthesis of DPN- d_4 .

Table 1

Method validation data for total and aglycone DPN and PPT in spiked rat serum.

ER Ligands	Expected concentration (ng/ml)	Day 1 Mean $\pm$ SD (RSD) (ng/ml)	Day 2 Mean $\pm$ SD (RSD) (ng/ml)	Accuracy (% added)
Aglycones				
PPT	0.5	0.584 $\pm$ 0.002 (0.4%)	0.539 $\pm$ 0.018 (3.4%)	112
	5	5.097 $\pm$ 0.008 (0.1%)	4.989 $\pm$ 0.125 (2.5%)	100
DPN	0.5	0.569 $\pm$ 0.028 (5.0%)	0.552 $\pm$ 0.022 (4.1%)	112
	5	4.738 $\pm$ 0.124 (2.6%)	4.968 $\pm$ 0.137 (2.7%)	97
Totals				
PPT	5	5.151 $\pm$ 0.095 (1.8%)	5.563 $\pm$ 0.269 (4.8%)	107
	25	25.401 $\pm$ 0.193 (0.7%)	28.020 $\pm$ 0.894 (3.1%)	107
DPN	5	4.353 $\pm$ 0.275 (6.3%)	4.520 $\pm$ 0.163 (3.6%)	89
	25	23.584 $\pm$ 1.115 (4.7%)	23.537 $\pm$ 1.532 (6.5%)	94

Control rat serum containing undetectable amount of PPT and DPN was spiked with PPT and DPN at two levels and processed on two different days, as described in the Experimental section and analyzed by UPLC/MS/MS. The mean PPT and DPN concentration (n = 4) are shown with the respective SD and relative standard deviation (RSD, %). The accuracy compares all measurements with the concentrations added.

\$watermark-text

\$watermark-text

\$watermark-text

Table 2

Pharmacokinetic parameters for DPN and PPT (2 mg/kg bw each) in Long-Evans rats (mean $\pm$ SD, n = 3) following a single subcutaneous injection. Measurements of total DPN and PPT were made after complete enzymatic hydrolysis using a mixture of β -glucuronidase and arylsulfatase and aglycone measurements were made without the enzymatic treatment.

	$t_{1/2 \text{ Elim}}^I$ (h)	$t_{1/2 \text{ Dist}}^I$ (h)	C_{max}^I (μM)	Fraction C_{max} unconjugated	AUC ($\mu\text{mol} \times \text{h} \times 10^{-1}$) ^I	Fraction AUC unconjugated
DPN-aglycone	4.6 $\pm$ 1.6	0.6 $\pm$ 0.4	1.0 $\pm$ 0.44	0.36 $\pm$ 0.05	2.5 $\pm$ 0.52	0.13 $\pm$ 0.02
DPN-total	8.2 $\pm$ 1.7	--	2.7 $\pm$ 0.90	--	19 $\pm$ 2.1	--
PPT-aglycone	3.2 $\pm$ 0.90	0.8 $\pm$ 0.6	0.35 $\pm$ 0.04	0.75 $\pm$ 0.10	2.0 $\pm$ 0.35	0.69 $\pm$ 0.04
PPT-total	6.0 $\pm$ 0.03	--	0.46 $\pm$ 0.03	--	2.9 $\pm$ 0.42	--

^I $t_{1/2 \text{ Elim}}$ = half-time for terminal elimination phase; AUC_{0- ∞} = area under the time-concentration curve from 0-infinity; C_{max} = maximal observed serum concentration.