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Pharmacokinetics of raloxifene in male Wistar–Hannover rats: Influence of complexation with hydroxybutenyl-beta-cyclodextrin

Michael F. Wempe [a,b,*], Vincent J. Wacher [c], Karen M. Ruble [a], Michael G. Ramsey [a], Kevin J. Edgar [d], Norma L. Buchanan [a], Charles M. Buchanan [a]

[a] *Eastman Chemical Company, Kingsport, TN, USA*
[b] *Department of Pharmacology, East Tennessee State University, Johnson City, TN, USA*
[c] *1042 N. El Camino Real, Suite B-174, Encinitas, CA 92024-1322, USA*
[d] *Wood Science & Forest Products, Virginia Tech, 230 Cheatham Hall, Blacksburg, VA 24061, USA*

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Abstract

Raloxifene is a highly insoluble, highly metabolized serum estrogen receptor modulator approved for use in the treatment of osteoporosis. Hydroxybutenyl-beta-cyclodextrin (HBenBCD) is a novel solubility enhancer previously demonstrated to increase the oral bioavailability of tamoxifen, letrozole, and itraconazole. The current study evaluated the pharmacokinetics of raloxifene in oral and intravenous formulations with HBenBCD in male Wistar–Hannover rats. Analytical methodology to measure raloxifene and its metabolites was developed by measuring raloxifene metabolism *in vitro*. Formulation with HBenBCD significantly increased raloxifene oral bioavailability. Mean ± S.D. oral bioavailabilities were 2.6 ± 0.4% for raloxifene formulated with microcrystalline cellulose, 7.7 ± 2.1% for a solid capsule formulation of raloxifene:HBenBCD complex, and 5.7 ± 1.3% for a liquid-filled capsule formulation containing raloxifene:HBenBCD/PEG400/H_2O. Relative to raloxifene/microcrystalline filled capsules, the presence of HBenBCD in the solid capsule formulation afforded: (i) a decrease in raloxifene T_{max} (2.5 ± 0.5 h versus 4.0 ± 0.5 h); (ii) a two-fold increase in raloxifene C_{max} and a three-fold increase in raloxifene AUC; and (iii) a 12-fold increase in raloxifene glucuronide C_{max} and a 6.5-fold increase in raloxifene glucuronide AUC. Hence, these studies demonstrate that raloxifene formulations containing HBenBCD significantly increased the oral bioavailability in rats relative to formulations that did not contain HBenBCD.

Keywords: Hydroxybutenyl-beta-cyclodextrin; Raloxifene; Pharmacokinetic studies; Bioavailability; Dissolution; Liquid chromatography–mass spectrometry/mass spectrometry

1. Introduction

Many drugs developed by the pharmaceutical industry suffer from poor water solubility (Wong et al., 2006; Naseem et al., 2004) which may substantially limit bioavailability. Improving oral bioavailability may reduce variability in systemic drug levels and effect (Rowland and Tozer, 1994). This may enable dose reduction leading to reduced drug side effects and expense. Therefore, developing drug delivery systems that increase solubility, dissolution rate, and improve bioavailability has been an important undertaking in pharmaceutical development. Various drug delivery techniques have been developed to overcome these limitations, such as (i) pro-drugs, (ii) addition of surfactants, (iii) salt selection, (iv) particle size reduction, and (v) inclusion complexes with cyclodextrins (CD) (Stahl and Wermuth, 2002; Malmsten, 2002).

Abbreviations: CD, cyclodextrin; HBenBCD, hydroxybutenyl-beta-cyclodextrin; LC–MS/MS, liquid chromatography–mass spectrometry/mass spectrometry; HPLC, high performance liquid chromatography; p.o., oral administration; i.v., intravenous administration; LOD, limit of detection; HPBCD, hydroxypropyl-beta-cyclodextrin; MS, molar substitution; S_o, solubility in a medium in the absence of a CD; S_t, solubility in a medium in the presence of a CD; C_{max}, means maximum plasma concentration; T_{max}, means time required to reach C_{max}; AUC, means total area under the plasma concentration-time; $AUC_{0\rightarrow 72}$, means total area under the plasma concentration-time curve from 0 to 72 h

* Corresponding author at: Eastman Chemical Company, Kingsport, TN, USA. Tel.: +1 423 224 7297; fax: +1 423 229 4558.
E-mail address: mwempe@eastman.com (M.F. Wempe).

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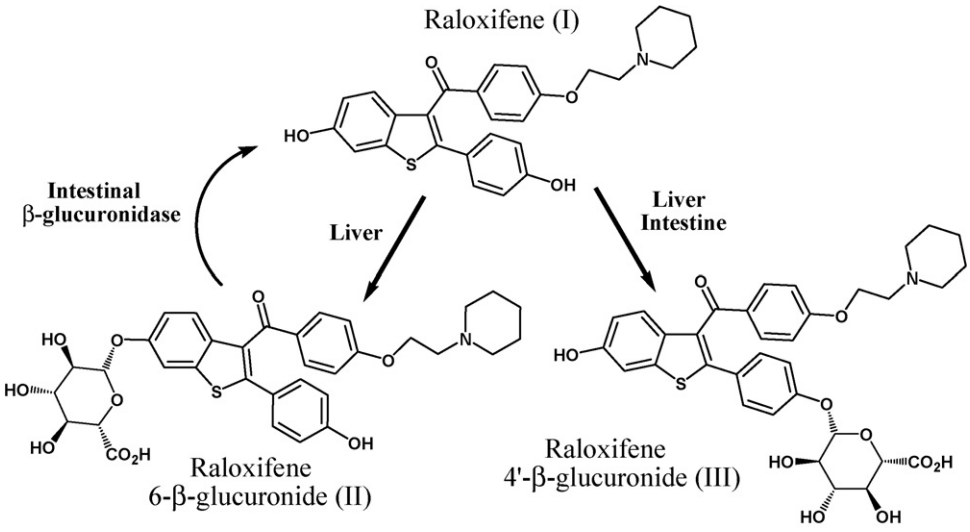

Fig. 1. Raloxifene glucuronidation.

CDs are cyclic glucose oligomers connected via α-1,4 linkages. Commonly used natural CDs contain 6, 7, or 8 glucose monomers and are typically referred to as α-CD, β-CD, and γ-CD, respectively. CDs form a torus and most CDs and CD derivatives have a hydrophobic interior and a hydrophilic exterior. CDs have an intrinsic ability to form specific inclusion complexes (Hirayama and Uekama, 1999; Uekama et al., 1998; Connors, 1997; Szejtli, 1991, 1995) and their unique physicochemical characteristics allow for exploitation in various applications (Sternbach and Rossana, 1982; Rideout and Breslow, 1980). The use of CDs in pharmaceutical formulations has been shown to enhance drug stability, solubility, and/or bioavailability (Loftsson et al., 1991, 2004; Redenti et al., 2000; Buchanan et al., 2006, 2007a–c; Wempe et al., 2007). Modified CDs, such as hydroxypropyl-beta-cyclodextrin (HPBCD), have been used in clinical formulations to overcome poor solubility issues and enhance bioavailability (Barone et al., 1998). Despite these successes, it is important to recognize that each particular CD has relatively narrow inclusion selectivity and that no one CD is a blanket solubility or bioavailability enhancer for all drugs. The restricted number of commercial alternatives to the currently available CD products makes it imperative to investigate novel CDs and determine their ability to enhance solubility, dissolution, and bioavailability. Previous work from our laboratories described the preparation and characterization of just such a novel CD, the highly water-soluble hydroxybutenyl-β-cyclodextrin (HBenBCD) (Buchanan et al., 2002).

In 1997, raloxifene hydrochloride (Evista®, an Eli Lilly product), a selective estrogen receptor modulator (SERM), was approved by the Food and Drug Administration as a treatment for osteoporosis. Raloxifene is a bone and liver estrogen agonist, which increases bone mineral density and decreases low-density lipoprotein (LDL)-cholesterol. In addition, raloxifene has been found to be a breast and uterus estrogen receptor antagonist and thereby may decrease the risk of invasive breast cancer. Raloxifene is highly insoluble in water and orally administered raloxifene undergoes rapid absorption and extensive first-pass metabolism (Hochner-Celnikier, 1999). As represented in Fig. 1, raloxifene (**I**) is metabolized via UDP-glucuronosyl-transferases (UGTs) to afford glucuronides (**II** and **III**); UGTs are membrane-bound proteins found in the endoplasmic reticulum. Raloxifene glucuronides are excreted into the intestine via bile (Czock et al., 2004), converted back to **I** via intestinal β-glucuronidase, and reabsorbed or excreted in feces. Raloxifene plasma concentration time profiles show multiple peaks, consistent with significant enterohepatic recycling (Morello et al., 2003). Clinical studies have revealed that the absolute bioavailability of raloxifene in humans is 2% and that glucuronide **III** is the major metabolite in plasma (Hochner-Celnikier, 1999). Raloxifene glucuronides **II** and **III** have been previously synthesized and competition experiments with ^{3}H-17β-estradiol for *in vitro* receptor binding suggest these metabolites are about 100-fold weaker in potency than raloxifene (Dodge et al., 1997). It is also well known that humans possess tissue-dependent UGT isoform distribution (Tukey and Strassburg, 2000). For example, human liver contains UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7, 2B10, 2B11, and 2B15 while 1A5, 1A7, 1A8, and 1A10 are absent. In contrast, human intestine contains UGT1A1, 1A4, 1A8, and 1A10 (Kemp et al., 2002). According to the investigations of Kemp et al., UGT1A8 and 1A10 – not present in human liver – appear to be the major contributors to raloxifene glucuronidation in jejunal microsomes. Furthermore, results from human liver microsomal incubations are not in agreement with those from clinical studies; that is, human liver microsomes were found to give slightly more **II** than **III** (Kemp et al., 2002). Raloxifene also undergoes extensive glucuronidation and sulfation in Caco-2 cells *in vitro* (Jeong et al., 2004).

Work from our laboratories has shown that complexation with HBenBCD was highly effective in enhancing tamoxifen and letrozole solubility (Buchanan et al., 2006; Wempe et al., 2007) and bioavailability in a rat model (Buchanan et al., 2006, 2007a,c). Since raloxifene is also a SERM, with water solubility issues, we chose to investigate whether complexation with

HBenBCD could enhance raloxifene solubility and bioavailability. Herein, we describe the preparation, isolation, *in vitro* solubility and dissolution testing of solid and liquid raloxifene:HBenBCD complex formulations, and pharmacokinetic studies in male Wistar–Hannover rats. Prior to conducting *in vivo* work, we needed a method for monitoring raloxifene and raloxifene metabolites. We also describe *in vitro* metabolism studies that were necessary to understand raloxifene absorption from HBenBCD complexes *in vivo*.

2. Materials and methods

Saquinavir base (Lot #25449; > 99% purity) and raloxifene hydrochloride (Lot #24552; ≥ 99% purity) were purchased from Apin Chemicals Ltd. (Abingdon, Oxon, UK). Raloxifene 6-β-glucuronide (Lot # 19-WG-9-1; > 99% purity) and raloxifene 4′-β-glucuronide (Lot #18-WG-171-1; > 99% purity) were purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Bulk plasma from Wistar–Hannover rats (containing potassium EDTA anti-coagulant) was obtained from Bioreclamation Inc. (Hicksville, NY). HPLC grade water, HPLC grade methanol, HPLC grade acetonitrile, ethanol, isopropyl alcohol, ammonium acetate, formic acid, polyethylene glycol 400 (PEG400), potassium hydroxide, hydrochloric acid, propylene glycol, nicotinamide adenine dinucleotide phosphate (NADPH), alamethicin, uridine-5′-diphospho-α-D-glucuronic acid (UDPGA), glutathione (GSH), *N*-Acetyl-L-cysteine (NAC), sulfur trioxide trimethyl amine complex, and hydroxypropyl-beta-cyclodextrin (HPBCD, MS = 4.4) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Hydroxybutenyl-beta-cyclodextrin (HBenBCD, MS = 4.7) was prepared according to the general methods previously described (Buchanan et al., 2002). Microsomes were obtained from two commercial sources: (i) mouse (male CD-1; Lot BDS), rat (male Fischer 344; Lot AJC), rat (male Wistar; Lot TCF), rat (male Sprague–Dawley; Lot LOL), dog (male beagle; Lot LQU), and monkey (male cynologous, Lot BYE) liver microsomes were purchased from *In Vitro* Technologies (Baltimore, MD); and (ii) human liver microsomes (mixed gender, pool of 10; Lot 0510007), rat intestinal microsomes (pool of 150; Lot 0510254), and human intestinal microsomes (pool of 8; Lot 0510228) were purchased from Xenotech, LLC (Lenexa, Kansas).

2.1. Equilibrium solubility determination of raloxifene

The equilibrium solubility of raloxifene in water was determined in the absence and in the presence of HPBCD, and HBenBCD (0–25 wt.%). Raloxifene equilibrium solubility in the presence of HBenBCD was also determined in water containing 3, 6, or 8 wt.% PEG400 as a co-solvent. Water was filtered through a Milli-Q Water System (Millipore Corporation, Bedford, MA). All CDs were dried at 10–15 mm Hg at room temperature for 14–60 h prior to use. Raloxifene (ca. 5–10 mg) was added to each well of a 2-mL 96-well polypropylene mixing plate, followed by either water or the appropriate CD solution (300–500 μL). After stock solution additions, the plate was sealed and shaken (Helidolph Titramax 1000) at 800–1200 rpm at 23 ± 2 °C for 48–72 h. Samples were transferred to a 96-well 2 mL multiscreen filter plate and filtered using a vacuum manifold. Raloxifene concentration was determined using UV spectroscopy. The UV measurements for raloxifene were made at 290 nm, with 350 nm used as a baseline point, and separated from the absorbance of HBenBCD (<200 nm). Raloxifene sample solutions (10–20 μL) were transferred to a 96-well plate (UV-STAR plates; Greiner, 190–400 nm spectral range) and diluted with 1:1 water:ethanol to afford an absorbance reading that was in the linear response range. Measurements were made using a SpectraMax Plus 384 Molecular Devices multiwell plate reader. Absorbance was then converted to raloxifene concentration. Each determination was done in triplicate. Blanks were used to determine the intrinsic solubility (S_o) of raloxifene in the corresponding solution, while the wells containing CD solutions were used to determine the solubility of raloxifene due to CD (S_t).

2.2. Raloxifene:HBenBCD solid powder complexes

Raloxifene hydrochloride (1.5 g) was added to a solution of HBenBCD (10.0 g) in 70 mL of water. The mixture was vortex-mixed and placed on a rotary shaker at 27 °C (230 rpm) for ca. 48 h. At the end of this mixing period, the pH of the water was 5.9. To maximize solubility, the pH was adjusted to ca. 3.0 with 0.2 N HCl. Following the pH adjustment, the sample was returned to the shaker for 22.3 h. Excess raloxifene was removed by filtration of the mixture through a 0.45 μm filter into a freeze dry flask. After freeze-drying, HPLC analysis established that the resulting white powder contained 12.2 wt.% raloxifene.

2.3. Raloxifene:HBenBCD PEG400 solution

A 20 wt.% solution of KOH in PEG400 (1.12 g) was added to a solution of HBenBCD (6.12 g) dissolved in PEG400 (4.05 g; dried over 4 Å molecular sieves). After mixing thoroughly, raloxifene hydrochloride (1.30 g) was added. The mixture was then vortex-mixed and placed in an ultrasonic bath until free of suspended particles. The pH of this solution was adjusted from ca. 8.7 to 8.2 by the addition of a HCl/propylene glycol solution (200 μL); therefore, the propylene glycol content was ≤2%. HPLC analysis revealed that this mixture contained 10.1 wt.% raloxifene. It is important to note that the raloxifene salt has poor solubility in the PEG400 solution, so adjustment of the solution pH to ca. 8.5 with KOH is necessary in order to obtain a solution with higher raloxifene concentrations.

2.4. In vitro dissolution studies

Raloxifene, raloxifene: HBenBCD, and raloxifene:HBenBCD/PEG400 solution (raloxifene = 15.3 ± 2.6 mg) were filled into hard shell Torpac Lock ring gel (size 0) capsules (Torpac, USA) using a filling funnel. *In vitro* dissolution testing was performed in triplicate (each formulation, $n = 3$) using a Varian VK 7000 dissolution tester (Cary, NC) according to method USP 28-NF 23 711 (United States Pharmacopeia, 2004)

with buffer solutions (900 mL) maintained at 37 °C and stirred at 50 rpm.

2.5. Animals

In vivo testing was conducted at RCC Ltd. (Toxicology, CH-4452 Itingen, Switzerland). Male Wistar–Hannover rats (weight range, 257–313 g) were obtained from RCC Ltd. (Laboratory Animal Services, CH-4414 Füllinsdorf, Switzerland). Prior to dosing, rats were individually housed in Makrolon type-3 cages with wire mesh tops and standardized softwood bedding (Lignocel Schill AG, CH-4132 Muttenz/Switzerland). The room was air-conditioned with 10–15 air changes per hour, and maintained at 22 ± 4 °C with a relative humidity between 30 and 70%. The rats were subjected to 12 h fluorescent light/12 h dark cycles with music during the light period. The animals were allowed free movement and access to water. Access to food was managed as described in the study design.

2.6. Pharmacokinetic study design

As summarized in Table 1, seven groups of either three or four male Wistar–Hannover rats (300–350 g) were administered different oral or intravenous formulations of raloxifene with or without HBenBCD. Oral dosage forms comprised capsules, containing the indicated raloxifene formulation in gelatin Torpac Lock ring gel size 9 capsules (Torpac, USA). Groups 1 (i.v.) and 4 (oral aqueous gavage) were dosed using a 1.0 mL syringe with 0.01 mL measurement capability. Group 1 was dosed at an infusion rate of 0.30 mL per min and group 4 was dosed as a 1.0 min bolus. Groups 2, 3, and 5–7 were each dosed by oral gavage; the capsule was followed by 0.50 mL of water to facilitate movement to the stomach. Animals were allowed free access to food and water, except that they were fasted for at least 8 h prior to dosing until 5 h post-dose. Dosing was 2–3.5 h after the beginning of a light cycle and dosing time across each group was consistent to avoid confounding chronopharmacokinetic effects.

Blood samples (300 μL) were collected from three or four animals/group/time point from a catheter inserted into the jugular vein using an AccuSampler® (DiLab®; Oresund, Sweden). For group 1 (i.v.), blood samples were taken at 0.083 (5 min), 0.25 (15 min), 0.50 (30 min), 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 12, 24, 36, 48, and 72 h. For group 4 (oral solution), blood samples were taken at 0.16 (10 min), 0.33 (20 min), 0.50 (30 min), 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 12, 24, 36, 48, and 72 h. For groups 2, 3, and 5–7 (oral capsules), blood samples were taken at 0.33 (20 min), 0.50 (30 min), 0.75 (45 min), 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 12, 24, 36, 48, and 72 h. After each blood draw, removed blood volume was replaced by an equivalent volume of intraperitoneal saline. Blood samples were centrifuged at RCC Ltd. and plasma transferred into a designated well of a 96-well plate. The plates were stored on dry ice during filling and shipped frozen on dry ice. All animals were euthanized 72 h post-dose following terminal blood collection via abdominal aorta or cardiac puncture. Animals were anesthetized by CO_2/O_2 for collection followed by exsanguination. On termination, livers were removed and flash frozen with liquid nitrogen. The liver samples were also stored and shipped frozen on dry ice; upon receipt, all samples were kept frozen (−80 ± 10 °C) until sample preparation and assayed using the validated LC–MS/MS method described below.

2.7. Determination of raloxifene and metabolites

2.7.1. Equipment

Plasma samples were analyzed for raloxifene and its glucuronide metabolites using a Sciex 4000-QTrap mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped

Table 1
Dosing groups for the raloxifene–HBenBCD pharmacokinetic study

Group	Dose (mg/kg)[a]	Description
1	i.v. (2.5)	Raloxifene:HBenBCD complex (122 mg raloxifene/g of solid powder) was dissolved in water to give a final concentration of 60 mg/mL (equivalent to 7.32 mg/mL raloxifene)
2	Oral, solid capsules (10)	Gelatin capsules containing raloxifene dispersed in microcrystalline cellulose (equivalent to 400 mg raloxifene base/g of solid powder)
3	Oral, solid capsules (10)	Gelatin capsules containing raloxifene:HBenBCD complex (122 mg raloxifene/g of solid powder)
4	Oral, aqueous gavage (10)	Raloxifene:HBenBCD complex (122 mg raloxifene base/g of solid powder) was dissolved in water to give a final concentration of 40 mg/mL (equivalent to 4.88 mg/mL raloxifene)
5	Oral, liquid capsules (10)	Gelatin capsules containing a solution of raloxifene (103 mg raloxifene/g liquid) and HBenBCD (400 mg/g) in PEG400/PG
6	Oral, liquid capsules (10)	Gelatin capsules containing a solution of raloxifene (equivalent to 100 mg raloxifene/g liquid) and HBenBCD (400 mg/g) in propylene glycol
7	Oral, liquid capsules (10)	Gelatin capsules containing a solution of raloxifene in propylene glycol and aqueous KOH (equivalent to 100 mg raloxifene/g liquid)

[a] All doses are based upon raloxifene base.

with a Shimadzu HPLC, a PEAK Scientific API Systems gas generator (Bedford, MA, USA), and Leap auto-sampler (Carrboro, NC).

2.7.2. Extraction procedure, calibration, and quality control samples

An internal standard (IS) solution was freshly prepared in a 500 mL volumetric flask containing 1:1 (v/v) acetonitrile:methanol and 0.04 μM of saquinavir base. Individually, the 96-well plates were removed from the freezer (−80 ± 10 °C) and allowed to warm to ambient temperature (45–50 min). The *in vivo* plasma samples (50 μL) were transferred into separate 1.5 mL micro-centrifuge tubes. Total plasma volume was brought to 100 μL by adding (50 μL) male Wistar–Hannover plasma (potassium EDTA). Subsequently, 200 μL of IS solution was added, capped, mixed (5 s), and centrifuged at 13,200 rpm (10 min) using an Eppendorf minispin centrifuge (Hamburg, Germany). The supernatant (250 μL) was transferred into individual wells of a 96-well plate. The 96-well plate was sealed and centrifuged at 3000 rpm (10 min) at 10 °C (Labofuge 400 R Centrifuge). The 96-well plate was then placed into the auto-sampler cool-stack (6 °C) and analyzed via LC/MS/MS.

Raloxifene standard curve ($n = 4$ for each; blank, 0.22, 0.44, 2.2, 4.4, 22.0, 44.0, 219.7, 439.5, and 1318.5 ng/mL) and raloxifene quality control (QCs, Q1–Q5; 0.44, 4.4, 22.0, 219.7, and 1318.5) samples were prepared by the addition and complete mixing of 100 μL stock aqueous raloxifene solutions with 900 μL of Wistar–Hannover plasma obtained from RCC Ltd. Raloxifene 6-β-glucuronide and raloxifene 4′-β-glucuronide standard curves ($n = 4$ for each; blank, 0.07, 0.33, 0.65, 3.3, 6.5, 32.5, 65.0, 324.9, and 649.7 ng/mL) and raloxifene 6-β-glucuronide quality control samples (QCs, Q1–Q5; 0.33, 3.3, 6.5, 65.0, and 649.7) were prepared analogously to the method described above. Samples were frozen (−80 ± 10 °C). After 24 h, the standard curve samples were removed from the freezer (−80 ± 10 °C) and allowed to warm (40–45 min) to ambient temperature. Standard curve and quality control samples (100 μL) were processed using the same method as employed for the *in vivo* samples as previously described.

The chromatographic system consisted of a Shimadzu SCL-10A Controller, LC-10AD LC, and DGU-14A Degasser (Shimadzu Scientific Instruments Inc.; Norcross, GA) connected to the Sciex 4000-Qtrap. Analyst 1.4.1 was used for data acquisition. Prism 4.02™ software (GraphPad Software Inc.; San Diego, CA) was used for data analysis, graphing, and statistical analysis. Ten (10) μL aliquots of the extracted samples were injected onto a Zorbax extended-C18 50 mm × 4.6 mm, 5-micron 80 Å column (Agilent Technologies, UK). The column temperature was set at 40 ± 1 °C using a Temperature Control Module (Analytical Sales and Services; Pompton Plains, NJ). A binary solvent gradient was used: solvent A was a 10 mM ammonium acetate solution containing 0.1% formic acid and solvent B was a 50:50 mixture of methanol:acetonitrile. Using a flow-rate of 0.4 mL/min, the following gradient was used for the HPLC separations: 95% A for 1.0 min; brought to 95% B at 3.0 min and held for 2.5 min; brought back to 95% A at 6.25 min and held for 1.75 min (8 min total). Between samples, the auto sampler was washed with a mixture of acetonitrile:methanol:isopropanol:water (1:1:1:1) containing 0.1% formic acid (Little et al., 2006).

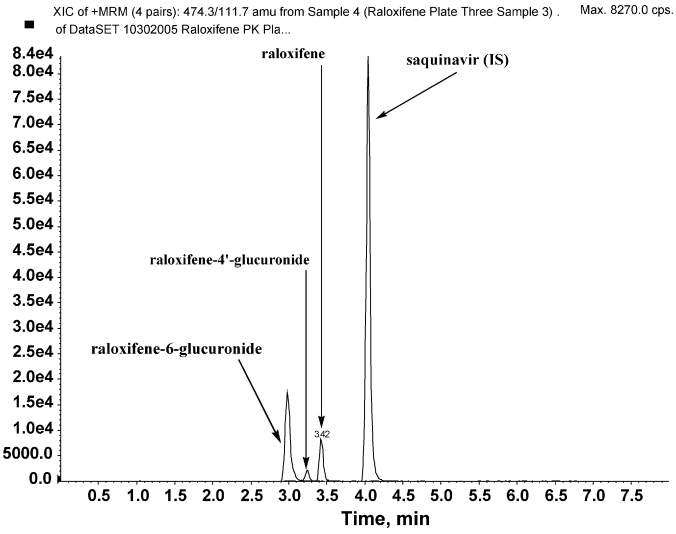

Fig. 2. Representative pharmacokinetic plasma chromatogram.

Raloxifene, raloxifene glucuronides, and saquinavir were analyzed using electrospray ionization operated in the positive mode (ESI+). The following mass spectrometer parameters were used: (i) an ion-spray voltage of 5500 V; (ii) temperature, 450 °C; (iii) nitrogen was used for the curtain gas (CUR) and for the Collisionally Activated Dissociation (CAD) gas; (iv) the CAD gas was set at medium; (v) Ion Source gas one (GS1) and two (GS2) were air and both set at 15.0; (vi) the entrance potential was set at 10.0; (vii) quadruple one (Q1) and three (Q3) were both set on Unit Resolution; (viii) dwell time was set at 200 ms; (ix) raloxifene, raloxifene glucuronides, and saquinavir were monitored using multiple reaction monitoring (MRM) employing a declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) of 80.0 V, 45.0 V, 8.0 V, and 80.0 V, 45.0 V, 8.0 V, and 61.0 V, 47.0 V, 14.0 V, respectively; and (x) mass transitions m/z 474.3 → 111.7 (raloxifene), m/z 650.3 → 474.2 (raloxifene glucuronides), and m/z 671.3 → 570.3 (saquinavir) were monitored (Fig. 2).

LC/MS/MS conditions for HBenBCD ($t_R = 2.5$–3.0 min) were the same as previously described except: (i) HPLC separation method was 95% A for 1.0 min; brought to 5% A at 3.0 min and held for 1.5 min; and brought back to 5% A at 5.0 min and held for 1.0 min (6.0 min total); (ii) temperature, 300 °C; (iii) CAD gas was set at high; (iv) GS1 and GS2 were set at 40.0; (v) Q1 was set on Open Resolution; (vi) DP, CE, and CXP of 71.0 V, 63.0 V, and 14.0 V, respectively; and (vii) mass transitions m/z 1432.5 → 233.1 (raloxifene) was monitored. The limit-of-detection (LOD) for HBenBCD was 1.5 ng/mL.

2.8. In vitro liver metabolite profile

To probe *in vitro* hepatic metabolism, liver microsomal incubations were conducted at 37.0 ± 0.1 °C and included (final concentration) 0.54 mg/mL microsomal protein with or without

various co-factors and/or reagents such as: NADPH (2.0 mM, cofactor for monooxygenases, e.g., cytochrome P450), UDPGA (8.0 mM, cofactor for glucuronidation; e.g. UGTs), GSH or NAC (5.0 mM, pH 7.4; to trap reactive metabolites), alamethicin (1.3 μg/mL) and 58.0 μM raloxifene (DMSO ≤ 0.2%, total v/v). Incubation components consisted of 100 mM phosphate buffer (pH 7.4), $MgCl_2$ (5.0 mM), and EDTA (1.0 mM).

Metabolism profile incubations were performed as follows: (1) 915 μL of a mixture consisting of potassium phosphate buffer, $MgCl_2$, NADPH, and/or UDPGA, and/or GSH/NAC and liver microsomal protein was pre-incubated at 37 ± 0.1 °C for 10 min; (2) the incubations were initiated by the addition and mixing of 120 μL test compound (500 μM), also pre-incubated at 37 ± 0.1 °C. After initiation (0.5 min), 10, 20, and 30 min, 200 μL of incubate was removed and added to quench solution (acetonitrile, 200 μL). The resulting samples were vortexed (5 s) and centrifuged at 3000 rpm (15 min) at 10 °C (Labofuge 400 R Centrifuge) and surveyed by LC/MS. The survey HPLC separation method (same solvent and flow as previously described) was as follows: 90% A for 1.0 min; brought to 60% A at 7.0 min and held for 0.5 min; brought to 5% A at 8.0 min and held for 2.9 min, and then brought back to 90% A at 13.0 min and held for 2.9 min (15.9 min total).

2.9. Synthesis of raloxifene–sulfate metabolites

To confirm that raloxifene sulfate metabolites were formed during *in vitro* metabolism experiments, an authentic sample was required and prepared as follows: A 10 mL round bottom flask containing a stir-vane was used. Under a N_2 environment, sulfur trioxide trimethyl amine complex (27.8 mg) was dissolved in pyridine (1.0 mL). Raloxifene hydrochloride (100 mg; 0.196 mmol) in pyridine (3.0 mL) was added via syringe transfer and stirred at room temperature (6 h). The reaction mixture was poured into diethyl ether (50 mL), mixed, cooled in an ice bath (10 min), and the bright yellow solid was filtered to afford a very hygroscopic solid which was dried under vacuum (0.3–0.4 mm Hg); crude raloxifene–sulfate, 108 mg.

2.10. Different species in vitro liver and intestinal microsomal incubation comparison

These *in vitro* incubations were conducted in triplicate at 37 ± 0.1 °C and included (final concentration) 1.0 mg/mL liver microsomal protein or 0.5 mg/mL of intestinal microsomal protein with UDPGA (4.4 mM, cofactor for glucuronidation; e.g. UGTs), alamethicin (25 μg/mL), and 10.0 μM raloxifene (DMSO ≤ 0.2%, total v/v). Incubation components consisted of 50.0 mM phosphate buffer (pH 7.4), $MgCl_2$ (5.0 mM), and EDTA (1.0 mM).

Incubations were performed as follows: (1) 420 μL of a mixture consisting of potassium phosphate buffer, $MgCl_2$, UDPGA, raloxifene, and microsomal protein were pre-incubated at 37.0 ± 0.1 (C for 10 min; (2) the incubations were initiated by the addition and mixing of 120 μL UDPGA, also pre-incubated at 37.0 ± 0.1 °C. After 1.0, 5.0, 15.0, 30.0, and 60.0 min, 100 μL of incubate was removed and added to quench solution (acetonitrile, 200 μL) containing saquinavir as an IS. The resulting samples were vortexed (5 s), centrifuged at 3000 rpm (10 min) at 10 °C (Labofuge 400 R Centrifuge), and analyzed by LC/MS/MS using the methods described for the *in vivo* analysis.

2.11. Liver sample preparation

The livers were removed from the freezer and placed onto dry ice. In group sets, each frozen liver was individually broken into pieces, weighed into a 50 mL centrifuge tube (Corning Inc., cat. #430828), two v/w of ice cold phosphate buffer (1X, pH 7.2; Gibco, cat. #20012–027) added, and homogenized (2 min) using a Polytron® PT1200 (Kinematica, CH; PT-DA 1212/2 EC). The samples were then stored frozen (−80 ± 10 °C). After allowing the samples to warm to ambient temperature, the samples were vortexed (5–10 s) and 500 μL aliquots (in triplicate) were transferred into individual 1.5 mL Eppendorf tubes. Subsequently, 500 μL of methanol/acetonitrile IS solution was added, capped, and vortex-mixed (10 s). The tubes were then centrifuged at 13,200 rpm for 10 min using an Eppendorf minispin centrifuge (Hamburg, Germany). The supernatant (830 ± 10 μL) was transferred into individual wells of a 96-well plate, sealed, and centrifuged at 3000 rpm (10 min) at 10 °C (Labofuge 400 R Centrifuge). The 96-well plate was then placed into the sample cool-stack (6 °C) and analyzed by LC/MS/MS.

2.12. Statistical methods

Statistical analysis on the effects of *in vitro* equilibrium solubility for different CDs were performed using a non-parametric Kruskal–Wallis (K–W) test followed by a Dunn's multiple comparison post-test (DMCPT) at a 95% confidence interval. The *in vitro* data for the equilibrium solubility in the presence of water containing PEG400 were analyzed using the Mann–Whitney (M–W) test with a two-tail p value test at the 95% confidence level. The effects of time and pH on drug solubility were analyzed using a Friedman's (F-T) test followed by a Dunn's multiple comparison post-test. The *in vivo* data were analyzed using the Mann–Whitney test with a two-tail p value test at the 95% confidence level. The formulation group comparisons, area Under the Curve (AUC), T_{max}, C_{max}, total exposure, and absolute bioavailability (F) were compared using a one-way ANOVA followed by a Dunn's multiple comparison test (significance level of $p < 0.05$).

3. Results and discussion

3.1. Bioanalytical pharmacokinetic summary

Prior to conducting *in vivo* experiments, it was prudent (e.g. for ethical reasons) to establish an effective Bioanalytical Pharmacokinetic (BAPK) method. As illustrated in Fig. 2, our LC/MS/MS method afforded retention times (t_R) of 2.9–3.1, 3.2-3.3, 3.4-3.5, and 4.0–4.2 min for raloxifene 6-β-glucuronide (**II**), raloxifene 4'-β-glucuronide (**III**), raloxifene (**I**), and the IS, saquinavir, respectively. To avoid endogenous lipid matrix ionization effects, in-source multiple reaction monitoring (IS-

Table 2A
Overall HPLC/MS/MS precision and accuracy for raloxifene

Sample	Theoretical conc. ng/mL	Observed raloxifene conc. ± S.D.	Accuracy	Precision
Q1	0.4	0.5 ± 0.1	125.0 ± 25.0	± 20.0
Q2	4.4	4.2 ± 0.8	95.5 ± 18.1	± 19.0
Q3	22.0	23.4 ± 3.3	106.4 ± 15.0	± 14.1
Q4	219.7	235.5 ± 24.4	107.2 ± 11.1	± 10.4
Q5	1318.5	1366.7 ± 129.8	103.7 ± 9.8	± 9.5

$n = 14$; accuracy is given as percent of the known value; precision is given as percent of the relative standard deviation.

Table 2B
Overall HPLC/MS/MS precision and accuracy for raloxifene 6-β-glucuronide

Sample	Theoretical conc. ng/mL	Observed raloxifene conc. ± S.D.	Accuracy	Precision
Q1	0.3	0.4 ± 0.1	133.3 ± 33.4	± 25.0
Q2	3.3	3.6 ± 0.5	109.1 ± 15.1	± 13.9
Q3	6.5	6.7 ± 0.7	103.1 ± 10.7	± 10.4
Q4	65.0	65.5 ± 4.4	100.7 ± 6.8	± 6.7
Q5	649.7	651.3 ± 27.3	104.4 ± 2.7	± 4.2

$n = 14$; accuracy is given as percent of the known value; precision is given as percent of the relative standard deviation.

MRM) was employed during method development (Little et al., 2006). Raloxifene, raloxifene glucuronides, and IS calibration data were fitted to a $1/x^2$ weighted (x = analyte concentration) linear regression using nine standard curve concentrations ranging from 0.3 to 1426 ng/mL (raloxifene) and 0.1–750 ng/mL (raloxifene glucuronides). The LOD for raloxifene, raloxifene 6-β-glucuronide, and raloxifene 4′-β-glucuronide were 0.22, 0.10, and 0.10 ng/mL, respectively. The correlation coefficients, computed using the Pearson correlation with a two tailed p value test ($p < 0.0001$) at a 95% confidence interval (data not shown), were 0.9995, 0.9996, and 0.9998, respectively. Five raloxifene and five raloxifene 6-β-glucuronide QC samples were used in this study and their relative accuracy and precision percentages are summarized in Tables 2A and 2B. These raloxifene accuracy and precision results are comparable to results others have reported for raloxifene (Zweigenbaum and Henion, 2000).

3.2. In vitro equilibrium solubility determination

Fig. 3 summarizes the relationships between the equilibrium solubility of raloxifene and the concentrations of the CDs. The two cyclodextrin derivatives were found to solubilize similar amounts of raloxifene and the differences were not statistically significant (K–W, $p = 0.96$; DMCPT, $p > 0.05$). For example, despite being derivatized with different functional groups, at 20 wt.% CD, each CD solubilized ca. 20 mg/mL of raloxifene.

The equilibrium solubility of raloxifene in water containing 0, 3, 6, and 8 wt.% PEG400 ($n = 3$ for each formulation) at 2 levels of HBenBCD (19 and 30%) was also investigated (data not shown). As might be expected, a higher concentration of raloxifene was achieved at 30 wt.% HBenBCD versus that achieved with 19 wt.% HBenBCD (M–W, $p = 0.0078$). For each HBenBCD group (19 and 30%), as the concentration of PEG400 increased, the amount of raloxifene solubilized by HBenBCD also increased; however, the differences were not statistically significant (DMCPT, $p > 0.05$). This example shows that PEG400 may be used as a co-solvent in the presence of HBenBCD and may slightly increase the solubility of raloxifene versus that obtained with HBenBCD alone. This was surprising, numerous studies have shown that co-solvents may compete with guest molecules for the cavity of a CD, thereby decreasing the solubility of the drug. In the case of polyethylene glycols, studies have also shown that PEG may form complexes with unmodified CDs and greatly reduce their solubility in aqueous media (Harada and Kamachi, 1990; Valero et al., 2003).

3.3. In vitro dissolution

The dissolution profiles of raloxifene and raloxifene:HBenBCD at pH 1.2, 4.5, and 6.8 are summarized in Fig. 4A. Raloxifene:HBenBCD showed a much faster *in vitro* dissolution rate than raloxifene. Comparing the two formula-

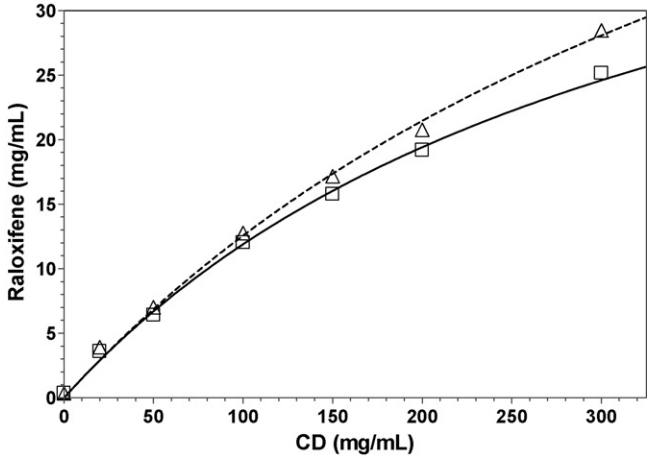

Fig. 3. Mean raloxifene equilibrium solubility concentration-CD profile 23 ± 2 °C (for each concentration and CD, $n = 3 \pm$ S.D.) fit to one-site binding are shown for (□) HBenBCD, and (△) HPBCD.

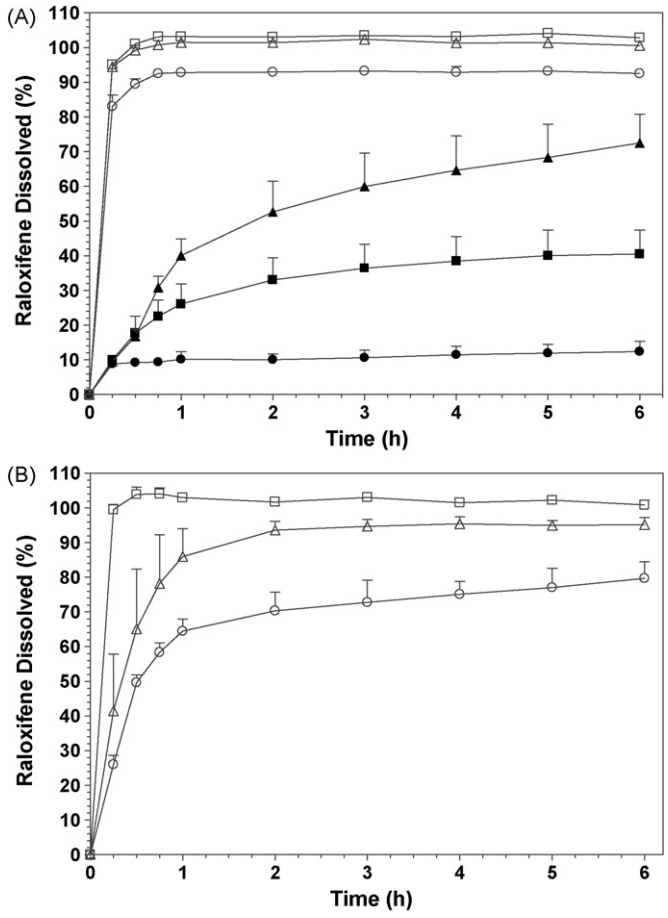

Fig. 4. (A) Mean dissolution profile of raloxifene formulations at 37 °C (each formulation type and pH, $n = 3 \pm$ S.D.). (□) raloxifene:HBenBCD pH 1.2; (△) raloxifene:HBenBCD pH 4.5; (◯) raloxifene:HBenBCD pH 6.8; (■) raloxifene pH 1.2; (▲) raloxifene pH 4.5; (●) raloxifene pH 6.8. (B) Mean dissolution profile of raloxifene:HBenBCD/PEG400 solution formulation at 37 °C (each pH, $n = 3 \pm$ S.D.). (□) pH 1.2; (△) pH 4.5; (◯) pH 6.8.

tions, the effects of time and pH on drug solubility were found to be significant (F-T, $p < 0.0001$; Gaussian approximation). Raloxifene was significantly more soluble in the presence than in the absence of HBenBCD at pH 1.2 and pH 6.8 (DMCPT, $p < 0.05$). Dissolution of raloxifene from raloxifene:HBenBCD powder filled capsules was rapid at each pH examined. Approximately 100% of the drug was released into the medium within 30 min at pH 1.2 and 4.5, and 90% was released at pH 6.8. Once dissolved, the drug did not crystallize; the solution concentration in the presence of HBenBCD remained constant over the time course of the experiment. In contrast, dissolution of raloxifene (no HBenBCD) was significantly slower with ca. 10–15% being dissolved after ca. 30 min. After 6 h, the maximum concentrations of raloxifene (no HBenBCD) reached were 12.5, 40.5, and 72.5% at pH 6.8, 1.2, and 4.5, respectively. These observations demonstrate that upon introduction of raloxifene:HBenBCD complexes into a simulated physiological environment, the raloxifene:HBenBCD formulations provide rapid release of raloxifene and stabilization of the resulting aqueous solution. Rapid release and higher concentration of drug translates to an increased oral bioavailability (*vide infra*).

The dissolution profiles of raloxifene:HBenBCD/PEG400 solution at pH 1.2, 4.5, and 6.8 are summarized in Fig. 4B. The effect of pH on drug solubility was found to be significant (F-T, $p < 0.0001$; exact). When formulated with PEG400 and HBenBCD, raloxifene was significantly more soluble at pH 1.2 than at pH 6.8 (DMCPT, $p < 0.001$). Dissolution of raloxifene was rapid at each pH examined. However, the extent of dissolution was pH dependent. Within 15 min, approximately 100% of the drug was released into the medium at pH 1.2, while release was only 41% at pH 4.5, and 26% was released at pH 6.8. Once dissolved, the drug did not crystallize. After 6 h, the maximum concentrations of raloxifene reached were 100, 95, and 79% at pH 1.2, 4.5, and 6.8, respectively. These observations also demonstrate that upon introduction of raloxifene:HBenBCD/PEG400 into a simulated physiological environment, the formulation afforded rapid release of raloxifene and stabilization of the resulting aqueous solution. Rapid release and higher concentration of drug translates to an increased oral bioavailability (*vide infra*).

Raloxifene is solubilized by HBenBCD via formation of a soluble complex. Upon freeze drying, the drug:CD complex is apparently maintained. Thermal analysis after freeze-drying (data not shown) of the complex indicates that raloxifene is amorphous and consistent with inclusion of the raloxifene in the HBenBCD cavity. The fact that both raloxifene and HBenBCD are both amorphous leads to an enhanced dissolution rate, and HBenBCD stabilizes the raloxifene thereby preventing crystallization. However, it should be noted that in the absence of HBenBCD, raloxifene is not soluble at an appreciable level. It is the formation of the drug:CD complex that leads to the amorphous state.

3.4. *In vitro microsomal incubations*

Prior to the analysis of the PK samples, it was prudent to investigate potential metabolites that may or may not be observed in the *in vivo* samples. For example, clinical samples from administration of ^{14}C-labeled raloxifene revealed only **I**, **II**, **III**, and raloxifene-6, 4′-diglucuronide suggesting that raloxifene is not metabolized by cytochrome P450 pathways (Hochner-Celnikier, 1999). However, recent *in vitro* evidence has been published illustrating that P450 oxidative pathways exist (Chen et al., 2002; Yu et al., 2004).

Various *in vitro* metabolites were chemically synthesized and/or detected from rat liver microsomal incubations and the results are summarized in Fig. 5. Overall, the current results are in agreement with previously published work (Chen et al., 2002; Kemp et al., 2002; Jeong et al., 2004; Yu et al., 2004) and illustrate that: (i) raloxifene glucuronidation is the major *in vitro* metabolic pathway, and (ii) oxidative pathways may occur, but are very minor. Under these *in vitro* incubation conditions, raloxifene-6-β-glucuronide (**II**) was formed much faster than raloxifene-4′-β-glucuronide (**III**); the ratio of **II**:**III** was 5:1. Only **I**, **II**, and **III** were observed in the *in vivo* plasma samples.

Recently, it was shown that rat intestinal microsomes produce raloxifene 6-β-glucuronide (**II**) as the major metabolite, while raloxifene 4′-β-glucuronide (**III**) was the predominant metabolite in female human intestinal microsomes (Jeong et

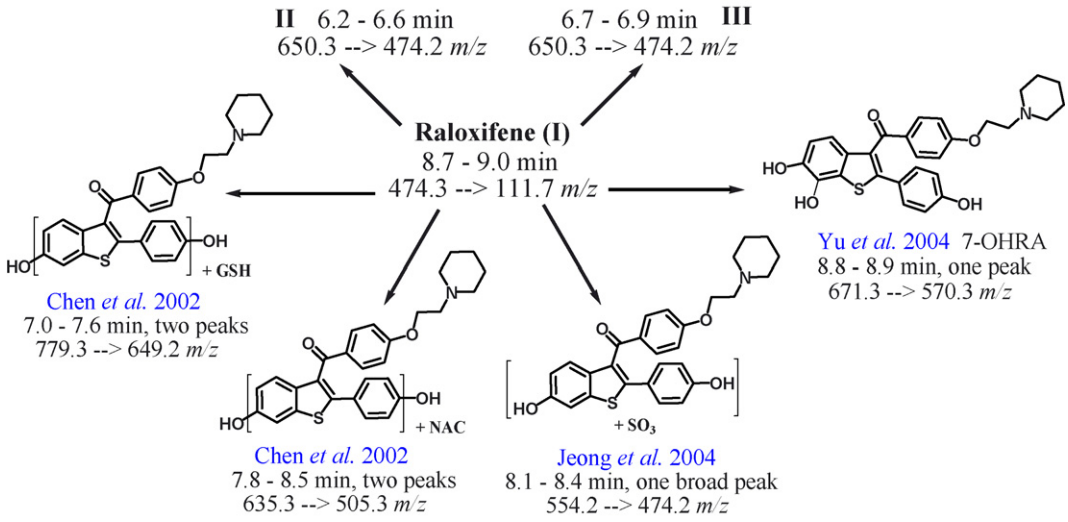

Fig. 5. Raloxifene *in vitro* rat hepatic metabolism.

al., 2005). Consequently, the total intrinsic clearance in human microsomes via raloxifene glucuronides was three-to-six-fold higher than in rats. These reported results prompted us to conduct an *in vitro* across-species (mouse, rat, dog, monkey and human) investigation. The observed rates of raloxifene glucuronide formation (**II** and **III**) appeared linear only up to 15 min. Therefore, the glucuronide ratios (**II**:**III**) at 15 min are summarized in Table 3. These data provide some interesting observations and conclusions under these incubation conditions. Firstly, rat liver microsomes, regardless of strain, provided a much higher rate of formation for raloxifene-6-glucuronide (**II**) than raloxifene-4′-glucuronide (**III**). The relative ratio of **III** produced via these liver microsomal incubations followed a species trend of rat < mouse < dog ≈ monkey < human. Finally, consistent with recent work (Jeong et al., 2005), human intestinal microsomes formed much higher ratios of **III** than did rat intestinal microsomes.

In considering these *in vitro* results, it is very important to emphasize that the source of microsomes, the protein content, the substrate, the co-factors present, their concentrations, and the overall general incubation conditions (for example, the amount of alamethicin used; Little et al., 1997) may alter the rate of glucuronide formation *in vitro*. This is highlighted by comparing the Wistar liver data from the *in vitro* metabolite profiling experiment (Fig. 5, ratio of **II**:**III** was 5:1) to the across-species comparison summarized in Table 3 (ratio of **II**:**III** was 1:0.6). The across-species incubations contained only cofactor UDPGA, had a lower substrate concentration, and the liver protein concentration was higher. Hence, *in vitro* incubations may provide qualitative predictions of *in vivo* results, but attempts to make quantitative comparisons may be perilous.

Table 3
Raloxifene glucuronide ratio across species, *in vitro*

Species	Organ	Relative ratio II:III
Mouse	Liver	1:1.6
Rat, Fischer	Liver	1:0.4
Rat, Wistar	Liver	1:0.6
	Liver	1:0.5
Rat, Sprague–Dawley	Intestine[a]	1:1.0
Dog	Liver	1:2.4
Monkey	Liver	1:2.2
	Liver	1:3.2
Human[a]	Intestine	1:6.5

[a] Microsomes from Xenotech LLC.

3.5. *In vivo oral absorption*

Plasma concentration versus time profiles for raloxifene and raloxifene glucuronides after intravenous or oral administration of raloxifene and raloxifene:HBenBCD formulations are illustrated in Figs. 6–8; for clarity, only the $AUC_{0–12}$ data are presented. Pharmacokinetic data for the raloxifene dosage forms are summarized in Table 4. Only **I**, **II**, and **III** were observed in the intravenous and oral *in vivo* plasma samples; the relative amount of **II** was always greater than **III**. In fact, the **II**:**III** ratio was consistently 9-10:1 in these *in vivo* samples. Hence, due to

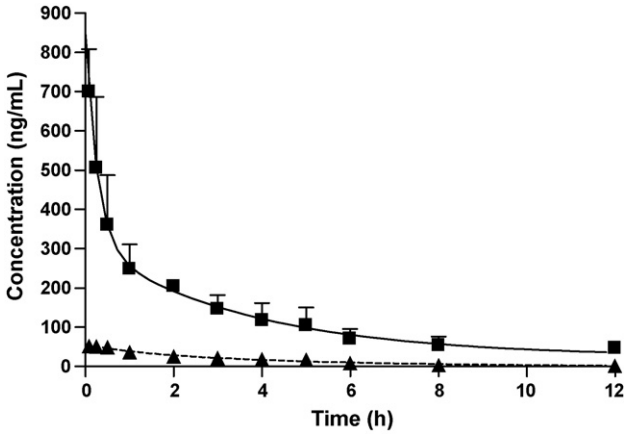

Fig. 6. Mean concentration (ng/mL) of raloxifene and raloxifene glucuronides from intravenous administration (2.5 mg/kg; group 1, each $n = 4 \pm$ S.D.) of raloxifene:HBenBCD. (■) raloxifene; (▲) raloxifene glucuronides.

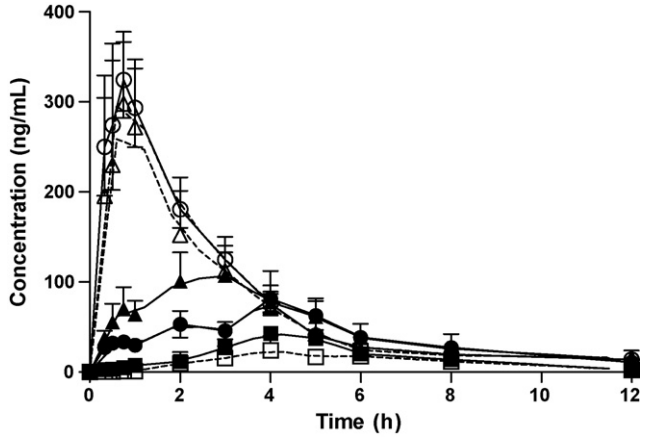

Fig. 7. Mean concentration (ng/mL; $n = 4 \pm$ S.D.) of raloxifene and raloxifene glucuronides from oral administration of solid raloxifene [group 2, (■) raloxifene, (□) raloxifene glucuronides]; raloxifene:HBenBCD capsules [group 3, (▲) raloxifene, (△) raloxifene glucuronides]; raloxifene:HBenBCD aqueous [group 4, $n = 3 \pm$ S.D., (●) raloxifene, (◯) raloxifene glucuronides].

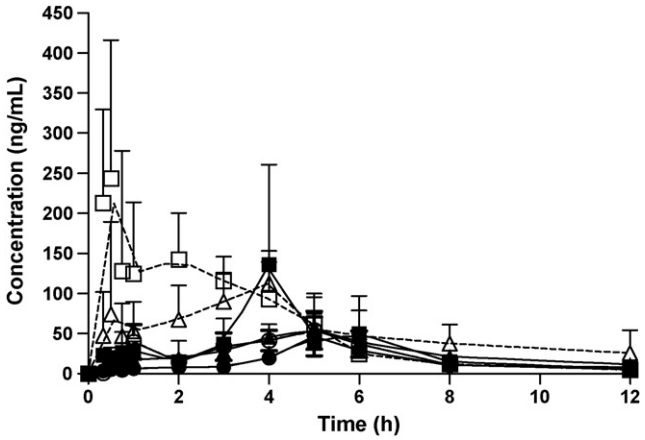

Fig. 8. Mean concentration (ng/mL) of raloxifene and raloxifene glucuronides from oral administration of raloxifene:HBenBCD/PEG400/PG liquid capsules [group 5; $N = 3 \pm$ S.D.), (■) raloxifene, (□) raloxifene glucuronides]; raloxifene:HBenBCD/PG liquid capsules [group 6; $N = 4 \pm$ S.D., (▲) raloxifene, (△) raloxifene glucuronides]; and raloxifene/PG liquid capsules [group 7; $N = 3 \pm$ S.D., (●) raloxifene, (◯) raloxifene glucuronides].

interspecies differences in glucuronidation (Walton et al., 2001), this data was also not consistent with the clinical data observed in humans as previously discussed.

Plasma concentration versus time profiles for raloxifene and raloxifene glucuronides after intravenous dosing of raloxifene:HBenBCD are shown in Fig. 6; the data fit a two-phase exponential decay. For simplicity, the AUCs for the raloxifene glucuronides (**II** and **III**) have been combined. The $AUC_{0-72\,h}$ for raloxifene dosed at 2.5 mg/kg (2965± 386 ng h/mL), and for the combined raloxifene glucuronides (182 ± 27 ng h/mL), indicate that raloxifene hepatic metabolism

Table 4
Raloxifene and Raloxifene:HBenBCD groups and summary of pharmacokinetic parameters

GRP	Formulation	Analyte	AUC_{0-72h} (ng h/mL)	T_{max} (h)	C_{max} (ng/mL)	Total exposure[a]	F[b]
1	HBenβCD Solution, iv	Raloxifene	11860 ± 1544[c]	<5 min	2800 ± 432[c]	1424 ± 165	100
		Glucuronides	728 ± 108[b]	0.25 ± 0.10	206 ± 43[c]		
	Oral						
2	Raloxifene (capsules)	Raloxifene	300 ± 41	4.0 ± 0.5	42.9 ± 4.2	55 ± 10	2.6 ± 0.4
		Glucuronides	153 ± 55	4.0 ± 0.5	24.3 ± 15.6		
3	Raloxifene:HBenBCD (capsules)	Raloxifene	901 ± 270**	2.5 ± 0.5**	107.6 ± 42.6 ns	231 ± 40**	7.7 ± 2.2**
		Glucuronides	1013 ± 130**	0.75 ± 0.25**	297.8 ± 80.0**		
4	Raloxifene:HBenBCD[d] (aqueous gavage)	Raloxifene	749 ± 27**	4.0 ± 0.5 ns	81.4 ± 25.7 ns	193 ± 18**	6.4 ± 0.8**
		Glucuronides	996 ± 153*		0.75 ± 0.25**	324 ± 73**	
5	Raloxifene/HBenBCD/ PEG400/PG[d] (liq. Fill capsules)	Raloxifene	668 ± 149*	4.0 ± 0.5 ns	136.3 ± 124.4 ns	192 ± 37**	5.7 ± 1.3*
		Glucuronides	887 ± 216*	0.50 ± 0.25**	243.5 ± 172.6*		
6	Raloxifene:HBenBCD/PG (liq. fill capsules)	Raloxifene	476 ± 154 ns	5.0 ± 0.5*	55.3 ± 40.0 ns	227 ± 80**	4.1 ± 1.2 ns
		Glucuronides	990 ± 648**	4.0 ± 0.5 ns	112.6 ± 40.7 ns		
7	Raloxifene/PG[d] (liq. fill capsules)	Raloxifene	321 ± 76 ns	5.0 ± 0.5 ns	46.5 ± 41.8 ns	94 ± 23 ns	2.7 ± 0.6 ns
		Glucuronides	391 ± 152 ns	5.0 ± 0.5*	54.3 ± 40.9 ns		

Groups 3–7 were compared to group 2. One-way ANOVA followed by a Dunn's multiple comparison test: ns = not significant.

[a] Total raloxifene exposure, (AUC raloxifene + AUC glucuronide)/raloxifene dose.

[b] Oral bioavailaility was calculated using the AUC_{0-72} for raloxifene only, not raloxifene + metabolites.

[c] AUC_{0-72} are normalized to a 10 mg/kg dose.

[d] ($N = 3$).

* p-Value <0.05.

** p-Value <0.01.

is not as significant (raloxifene glucuronides AUC/raloxifene AUC = 0.062 ± 0.012) as intestinal phase II metabolism after oral dosing (*vide infra*). After the distribution phase, the initial rates of elimination for both raloxifene and raloxifene glucuronides were rapid ($t_{1/2}$ 2.5 ± 0.3 h). After ca. 8 h, significant enterohepatic recycling causes the $t_{1/2}$ to become significantly longer.

Plasma concentration versus time profiles for raloxifene, and raloxifene glucuronides after oral dosing of raloxifene/microcrystalline filled capsules (group 2), raloxifene:HBenBCD powder filled capsules (group 3), and raloxifene:HBenBCD aqueous (group 4) are shown in Fig. 7. In the case of oral raloxifene capsule dosing (group 2), T_{max} values for both drug and metabolite were 4 h. Relative to i.v. dosing, phase II drug metabolism was far more significant ($p < 0.0001$) via oral administration (raloxifene glucuronides AUC/raloxifene AUC = 0.52 ± 0.20). When the animals were dosed with raloxifene:HBenBCD powder filled capsules (group 3), the $AUC_{0–72\,h}$ values for both raloxifene and raloxifene glucuronides were significantly ($p < 0.01$) larger than control (group 2). In addition, the T_{max} values for raloxifene and raloxifene glucuronides (group 3) were smaller than in group 2 and indicate a more rapid rate of absorption and metabolism in the presence of HBenBCD. After dosing the raloxifene:HBenBCD complex, raloxifene phase II metabolism (group 3 and 4) was statistically (both $p < 0.01$) more extensive (raloxifene glucuronides AUC/raloxifene AUC = 1.21 ± 0.41 and 1.33 ± 0.21, respectively) than control (group 2, no HBenBCD).

Plasma concentration versus time profiles for raloxifene and raloxifene glucuronides after oral dosing of raloxifene and raloxifene:HBenBCD liquid filled capsules (groups 5–7) are portrayed in Fig. 8. Dosing with a solution of raloxifene dissolved in propylene glycol containing no HBenBCD (group 7) afforded lower AUC values for raloxifene and raloxifene glucuronides than those of animals dosed with HBenBCD containing liquid formulations in either PEG400/PG (group 5) or PG (group 6) (Table 4). It is interesting to note that, relative to dosing with raloxifene/PG liquid filled capsules (group 7), the AUC for raloxifene (group 6) was essentially unchanged while the AUC for the raloxifene glucuronide was significantly increased. The pharmacokinetic parameters for the animals dosed with raloxifene and HBenBCD dissolved in PEG400/PG (group 5) were very similar to those obtained by dosing the animals with an aqueous gavage of a raloxifene:HBenBCD complex (group 4). However, when the animals were dosed with raloxifene and HBenBCD dissolved in propylene glycol (group 6), the pharmacokinetic parameters were different. In particular, T_{max} for raloxifene glucuronides increased to 4 h and the ratio of raloxifene glucuronides AUC/raloxifene AUC (2.27 ± 1.77) was higher.

3.6. HBenBCD from i.v. dose group one

In addition to raloxifene and raloxifene glucuronides, group 1 samples contained HBenBCD. Therefore, group 1 was also analyzed for HBenBCD absorbed into plasma (20 mg/kg HBenBCD dose). The HBenBCD plasma concentration versus time profile showed first-order elimination (data not shown); the log plot afforded an extrapolated concentration of $C_{(0)} \approx 10{,}257$ ng/mL. HBenBCD pharmacokinetics were calculated using standard pharmacokinetic equations (Rowland and Tozer, 1995). Clearance (CL) was 1.6 ± 0.2 L/h. It took 15 min for HBenBCD to distribute (distribution phase) and enter the elimination phase. During the elimination phase, the apparent volume of distribution for HBenBCD was 1.0 ± 0.2 L and the elimination half-life was 26.8 ± 4.8 min. It is also important to note that under the analytical methodology used, described in section 2.7, we did not observe any noteworthy ion-suppression effects.

3.7. In vivo liver samples

The *in vivo* liver sample data is summarized in Table 5. Even after 72 h, all of the livers showed evidence of raloxifene and raloxifene-glucuronide. It is interesting to note that groups containing HBenBCD with PEG400/PG and/or propylene glycol (groups 5, 6) had the highest liver concentrations of raloxifene. Within-group animal variability was evident and a few additional observations are notable. In group 3, rat liver N4 contained a high level of raloxifene, which paralleled much higher plasma concentrations in this animal. Similarly, in group 5, rat liver N1 had the highest raloxifene and raloxifene-glucuronide concentrations of all animals tested and these corresponded with

Table 5
Rat liver samples and drug/drug metabolite results

Group#	Liver sample	Raloxifene ng/g liver	Raloxifene-glucuronides ng/g liver
1	N1	17.0 ± 2.7	9.5 ± 3.9
1	N2	4.7 ± 0.3	0.7 ± 0.1
1	N3	6.4 ± 0.3	0.9 ± 0.3
1	N4	7.1 ± 1.7	1.2 ± 0.3
2	N1	7.4 ± 1.0	1.0 ± 0.1
2	N2	2.6 ± 0.3	0.4 ± 0.1
2	N3	3.5 ± 0.4	0.4 ± 0.3
2	N4	2.9 ± 0.1	0.4 ± 0.2
3	N1	5.7 ± 0.5	0.8 ± 0.1
3	N2	12.6 ± 2.1	1.1 ± 0.4
3	N3	4.7 ± 0.6	0.9 ± 0.2
3	N4	137.0 ± 2.9	2.2 ± 0.4
4	N1	22.3 ± 0.5	15.7 ± 3.1
4	N2	6.6 ± 0.2	0.9 ± 0.2
4	N3	20.8 ± 4.4	2.2 ± 0.4
5	N1	288.2 ± 5.4	73.3 ± 6.9
5	N2	48.6 ± 1.1	14.7 ± 1.0
5	N3	36.4 ± 1.3	4.3 ± 0.4
6	N1	36.1 ± 8.7	9.2 ± 1.8
6	N2	28.6 ± 1.7	6.2 ± 0.2
6	N3	63.1 ± 0.3	13.5 ± 1.3
6	N4	11.7 ± 3.2	2.6 ± 0.1
7	N1	42.4 ± 2.4	5.2 ± 0.5
7	N2	9.9 ± 0.5	2.9 ± 0.5
7	N3	2.0 ± 0.6	0.6 ± 0.2

high plasma concentrations. The time course of HBenBCD in the liver also differed between intravenous and oral dosing. In group 1 (iv), HBenBCD could not be measured in plasma 72 h post-dose; however, the livers clearly contained HBenBCD (377 ± 170 ng/mL per g liver). In contrast, no evidence of HBenBCD was observed in the plasma or the livers of animals in groups 2–7 (oral dosing) 72 h post-dose.

4. Conclusion

Raloxifene oral bioavailability (F) after dosing to male Wistar–Hannover rats with raloxifene powder filled capsules was $2.6 \pm 0.4\%$. When the animals were dosed with raloxifene:HBenBCD powder filled capsules, the oral bioavailability of raloxifene was $7.7 \pm 2.2\%$ (a three-fold increase). Likewise, the oral bioavailability of the other 3 HBenBCD formulations (groups 4–6) were $6.4 \pm 0.8\%$ (a 2.5-fold increase), $5.7 \pm 1.3\%$ (a 2.2-fold increase), and $4.1 \pm 1.2\%$ (a 1.6-fold increase), respectively.

Since raloxifene undergoes extensive presystemic metabolism, measurement of raloxifene levels alone may not provide the best indication of the extent of raloxifene dissolution and uptake from the intestine into the portal blood. A better measure of the effect of the HBenBCD on total raloxifene absorption may be 'total raloxifene exposure', measured as (AUC raloxifene + AUC metabolites)/raloxifene dose. Using this combined measure (Table 4), it can be seen that administration of raloxifene as raloxifene:HBenBCD powder filled capsules caused a 4.2-fold increase in total raloxifene systemic delivery.

If one compares the oral bioavailability of raloxifene formulated as a liquid (no HBenBCD, group 7) to that obtained with the other liquid fill formulations (groups 5 and 6), the importance of HBenBCD in maintaining raloxifene solubility was clearly evident; the oral bioavailability of raloxifene was 1.5–2.1 times greater and the total raloxifene exposure was twice that observed in the absence of HBenBCD. Clearly, these examples demonstrate HBenBCD's potential to substantially enhance oral raloxifene exposure in mammals.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2007.06.002.

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