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DISPOSITION OF LASOFOXIFENE, A NEXT GENERATION SELECTIVE ESTROGEN RECEPTOR MODULATOR, IN HEALTHY MALE SUBJECTS

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Running title: *In vivo* and *in vitro* metabolism of lasofoxifene

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[1]Abbreviations used are: SERM, selective estrogen receptor modulator; EPT, estrogen-progestin replacement therapy; ER, estrogen receptor; SRM, single reaction monitoring; SAM, S-adenosyl methionine; ABT, aminobenzotriazole; ICH, international conference on harmonization; GCP, Good Clinical Practices; MTBE, methyl-*tert*-butyl ether; COMT, catechol-O-methyltransferase; UGT, UDP-glucuronosyltransferase; UDPGA, UDP-glucuronic acid.

ABSTRACT

Disposition of lasofoxifene, a next generation selective estrogen receptor modulator, was investigated in male volunteers after oral administration of a single 20 mg dose of [^{14}C]lasofoxifene. Approximately 72% of the administered dose was recovered from the urine and feces, with majority of dose excreted in the feces probably via bile. The absorption of lasofoxifene in humans was slow with T_{max} values typically exceeding 6 h. The C_{max} and $AUC_{(0\text{-}tlast)}$ values of lasofoxifene were lower than those determined for total radioactivity, indicating presence of circulating metabolites. The primary clearance mechanisms for lasofoxifene in humans were direct conjugation (glucuronide and sulfate conjugates) and phase I oxidation, each accounting for about half of the metabolism. Several oxidative metabolites were identified by LC/MS/MS. The primary phase I metabolites were due to hydroxylations on the tetraline moiety and the phenyl rings attached to the tetraline, and oxidation on the pyrrolidine moiety. Considering the numerous metabolites seen *in vivo*, additional *in vitro* studies using human liver and intestinal microsomes, recombinant CYPs and UGTs were performed. The turnover of lasofoxifene was very slow in liver microsomes and only two metabolites were identified as two regioisomers of the catechol metabolite. The results from *in vitro* experiments with recombinant isoforms and CYP-isoform selective inhibitors suggested that the oxidative metabolism of lasofoxifene is catalyzed primarily by CYP3A and CYP2D6. In addition, its glucuronidation is catalyzed by UGTs that are expressed in both the liver (1A1, 1A3, 1A6 and 1A9), and the intestine (1A8 and 1A10).

Postmenopausal women have an increased risk of developing osteoporosis as their levels of endogenous estrogen decline (Luisetto et al., 1993; NIH 1994; Gambacciani et al., 2000; Riggs et al., 2002; Sowers et al., 2003a, 2003b). In the past, osteoporosis has been effectively treated with estrogen-progestin replacement therapy (EPT[1]), but recent findings from the Women's Health Initiative study have shown that the risks outweigh the benefits (Rossouw et al., 2002; Chlebowski et al., 2003; Manson et al., 2003; Hodis et al., 2003; Wassertheil-Smoller et al., 2003). The long-term use of EPT is no longer recommended except under special circumstances, although its short-term use for the relief of vasomotor and urogenital symptoms is considered acceptable. As a consequence, alternative long-term anti-osteoporotic therapies are being actively sought, and agents that can mimic the positive effects of estrogen without increasing the risk of breast or endometrial cancer are of particular interest.

Lasofoxifene (Fig. 1), a next generation SERM, is being developed for the prevention and treatment of osteoporosis. Studies have shown that lasofoxifene is an ER agonist in bone and cholesterol regulation, and an ER antagonist in the mammary gland and uterus. It binds with high affinity to the human ERα, has good oral bioavailability in preclinical species (Rosati et al., 1998), prevents bone loss, and maintains bone mass and strength in ovariectomized rats without stimulating the uterus (Ke et al., 1998, 2004). In both non-clinical and clinical human studies, lasofoxifene have shown a favorable safety profile and demonstrated a proven efficacy in preventing bone loss and lowering cholesterol levels (Gennari et al., 2006) and it is now under submission for regulatory approval.

It is important to understand how a new therapeutic agent is metabolized and excreted by the body. Metabolism and excretion studies in humans provide valuable information about any potential drug–drug interactions that might need to be considered when co-administering a

long-term treatment such as lasofoxifene with other drugs frequently used in postmenopausal women. A thorough understanding of the clearance mechanisms of lasofoxifene should also help to predict whether extra care, such as dose adjustment, will need to be taken when treating special patient groups, for example those with renal or liver disease. Preliminary *in vitro* studies suggested that lasofoxifene was stable in rat, monkey and human liver microsomes. These findings indicated that lasofoxifene is unlikely to undergo rapid oxidative metabolism *in vivo* in the rat, monkey, or human. However, *in vivo* studies in rats and monkeys have shown that lasofoxifene is metabolized by phase I oxidation and phase II conjugation reaction (Johnson and Prakash, 1998). The objectives of the present study were to evaluate the metabolism and excretion of radiolabelled lasofoxifene in healthy men, and to identify and characterize the enzymes (CYPs and UGTs) involved in the metabolism. The metabolites were characterized by LCMS/MS using a SRM technique and by comparison of their HPLC retention times with those of metabolites identified in preclinical species.

MATERIAL AND METHODS

General Chemicals.

Commercially obtained chemicals and solvents were of HPLC or analytical grade. Ecolite (+) scintillation cocktail was obtained from ICN (Irvine, CA). Carbosorb and Permafluor E+ scintillation cocktails were purchased from Packard Instrument Company (Downers Grove, IL). HPLC grade acetonitrile, methanol and water, and certified ACS grade ammonium acetate and acetic acid were obtained from J.T. Baker (Phillipsburg, NJ). SAM and isoform specific inhibitors, ketoconazole, quinidine, quercetin, sulfaphenazole, furafylline and ABT, were obtained from Sigma-Aldrich Chemicals (St. Louis, MO).

Human liver samples were obtained from 20 organ donors (both male and female between the ages of 18-65 years) and purchased from Tissue Transformation Technology (Edison, NJ)

upon an approval from ethics committee of the vendor. Liver and microsomal samples were stored at -70^{o}C until used. Human liver and intestinal microsomes and other subcellular fractions (S-9 and cytosol) were prepared and characterized for CYP isoforms at Pfizer Global Research & Development (Groton, CT) using standard procedures (Prakash et al., 2000). HL-mix 13 was prepared by mixing liver microsomes from several donors to represent the CYP in normal human. Recombinant human CYP isoforms (CYP1A1, CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5) and UGT isoforms (1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17) were purchased from Gentest (Bedford, MA). The microsomal protein was assayed using BCA assay kit (Pierce, Rockford, IL) and its P450 contents were determined by the method described previously (Omura and Sato, 1964).

Radiolabelled Drug and Reference Compounds

[^{14}C]Lasofoxifene [CP-336,156-CB (tartrate salt, Fig. 1)], uniformly labeled with C-14 at the phenyl ring attached to tetraline moiety, was synthesized by the Radiosynthesis Group at Pfizer Global Research and Development (Groton, CT). The radiochemical purity was ≥99%, as determined by HPLC using an in-line radioactivity detector and the specific activity was 1.93 mCi/mmol. [^{14}C] Lasofoxifene (specific activity; 32.5 mCi/mM) was used for in vitro studies. The synthetic reference compounds standards, 5-hydroxy- (M21A), 7-hydroxy- (M21B), 5-methoxy- and 5-hydroxy-6-O-methyl-, 7-methoxy- and 7-hydroxy-6-O-methyl-lasofoxifene were synthesized at Pfizer Global Research and Development (Groton, CT) using standard procedures (Rosati et al., 1998).

Subject and Dose Administration

Four healthy male subjects between the ages of 18 and 45 (inclusive) years participated in the study. All subjects provided written, informed consent prior to participation in the study. The study protocol, consent documents, consent procedures and subject recruitment

procedures were approved by the Independent Institutional Review Board. The study was conducted in compliance with the International Conference on Harmonization (ICH) Good Clinical Practices (GCP) guidelines, the ethical principles that have their origin in the Declaration of Helsinki, and in compliance with the US Food and Drug Administration regulations for informed consent and protection of patient rights. Subjects entered the Clinical Research Facility approximately 12 h before dosing, and remained there for at least 480 h after dosing under continuous medical observation. All subjects had fasted for at least 8 h prior to morning dosing. The subjects were given a single 20 mg dose of [^{14}C]lasofoxifene (~80 μCi/subject). The drug was administered in an open fashion as a single oral dose in the morning. A standard meal was provided 4 h later. The dosing solution was prepared by dissolving the radiolabelled drug in water (100 ml). Subjects were required to refrain from lying down during the first four hours after drug administration. Subjects were prohibited to consume caffeine-containing foods and/or beverages for 24 h prior to dosing and for 48 h after dosing. Administration of the study drug by or under the supervision of medical personnel ensured adherence.

Plasma, Urine and Feces Collection

Venous blood samples (enough to yield 6 ml of plasma) for lasofoxifene concentrations and total radioactivity were collected from each subject in heparinized glass vacuum blood collection tubes prior to dosing (0) and at 1, 2, 4, 8, 12, 16, 24, 36, 48, 72, 120, and 168 h following dosing. At 216, 264, 336, 408, and 480 h post dose, blood samples sufficient to yield 3 ml of plasma were collected. All blood samples were separated in a refrigerated centrifuge within 1 h of collection. Blood samples collected up to 168 h post dose were divided into two 3-ml aliquots and stored frozen at or below -70 ^{o}C. One 3-ml aliquot from

each time point was used for analysis of lasofoxifene concentrations. The remaining 3-ml sample was used for analysis of total radioactivity.

For the analysis of lasofoxifene metabolites, venous blood samples (sufficient to yield 20 ml of plasma) were collected from each subject as described above at 6, 24 and 48 h post dose administration. These were stored at or below -20 ^{o}C until analyzed.

Each subject emptied their bladder and provided a 50-ml sample of urine immediately prior to dosing, which was frozen at or below -20 ^{o}C. Urine was collected from 0-12, 12-24, and every 24 h intervals for 24 days. All of the urine collected during each time period was mixed, the total volume recorded, and an aliquot removed for storage at or below -20 ^{o}C until analysis. Fecal samples were collected as passed, from the time of dosing until at least 480 h after dosing.

Measurement of Total Radioactivity in Plasma, Urine and Feces

All measurements of total radioactivity were performed by liquid scintillation counting at Phoenix International Life Sciences Inc (Montreal, Ontario). Samples of plasma and urine (1 ml) from each sampling time point were added to 10 ml of Ecolite (+) scintillation fluid and counted in a liquid scintillation counter (Beckman, Fullerton, CA). Fecal samples were transferred into tarred stomacher 3500 bags, hydrated with equal amounts of water, and homogenized using a Stomacher homogenizer (Cooke Laboratory Products, Alexandria, VA). Triplicate aliquots (~0.25-0.5 g) of each fecal homogenate were weighed into oxidizer sample cups and combusted in an oxidizer (Model 307, Packard, Downers Grove, IL). The liberated $^{14}CO_2$ was trapped by Carbosorb E (Perkin Elmer Life and analytical Sciences, Boston, MA). Scintillation cocktail Permafluor V (Perkin-Elmer Life and analytical Sciences, Boston, MA) was added to the samples and counted in a Perkin-Elmer Wallac 1409 liquid scintillation

counter (Perkin-Elmer Life and Analytical Sciences). Combustion efficiency was determined by combustion of the ^{14}C-Spec Check standard in an identical manner. Radioactivity less than twice the background value was considered to be below the limit of determination. Fecal samples collected prior to dosing were used as the control samples and provided the background count rate.

The actual dose of radioactivity administered to each subject was determined by measuring the residual radioactivity in the dosing container following dose administration and subtracting this from the total radioactive dose in the dosing container. When determining the amount of radioactivity excreted in urine and feces at each time point as a proportion of the amount administered, the net radioactivity in the actual dose was considered to be 100%. The amount of radioactivity in plasma at each time point was calculated using the specific activity of the dose administered and was expressed as nanogram-equiv of parent drug per milliliter.

Pharmacokinetic Analysis

Concentrations of unchanged lasofoxifene in plasma were determined at CEDRA Corporation (Austin, TX, USA) using a validated LC/MS/MS assay. The analytical method was validated for analysis of lasofoxifene in 1 ml heparinized human plasma samples over a concentration range of 0.025-6.0 ng/ml. The pharmacokinetic parameters were determined using the WinNonlin-Pro Ver. 1.5 program (Pharsight; Mountain View, CA) by non-compartmental approach. The pharmacokinetic parameters for lasofoxifene and total radioactivity were calculated from the plasma concentration data. The apparent maximum plasma concentration (C_{max}) and the time at which this concentration was achieved (T_{max}) were taken directly from the concentration data. The area under plasma concentration–time profile from time zero to the last quantifiable time point, ($AUC_{0-tlast}$), was calculated using

linear trapezoidal approximation. The plasma terminal phase rate constant (K_{el}) was estimated using least square regression analysis of the plasma concentration–time data obtained during the terminal log-linear phase. Terminal elimination half-life ($t_{½}$) was calculated by $\ln 2/K_{el}$ relationship. The area under plasma concentration–time profile from time t_{last} to infinity ($AUC_{tlast-\infty}$) was estimated as C_{est}/K_{el}, where C_{est} represented the estimated concentration at the last time point time in which drug was quantitated based on the regression analysis. The $AUC_{0-\infty}$ was estimated as the sum of the $AUC_{0-tlast}$ and $AUC_{tlast-\infty}$ values. For the purpose of calculating mean drug concentrations at each sampling time, concentrations less than the LLOQ were considered to be 0 ng/ml; a mean was not calculated when 50% or more of the concentrations were <LLOQ.

Extraction of Metabolites from Biological Samples

Plasma samples (20 ml) taken from each subject at 24 and 48 h post dose administration were mixed with 40 ml of acetonitrile, vortexed and sonicated. The mixture was centrifuged and the supernatant collected. The remaining pellet was mixed with 20 ml of acetonitrile, centrifuged and the supernatant combined with the first supernatant. The overall recovery of radioactivity in plasma was about 78-85%. The combined supernatants were evaporated to dryness in nitrogen Turbo Vap LV evaporator (Caliper Life Sciences Corporate, Hopkinton, MA). The residue was reconstituted in 300 µl of methanol:water (1:1), centrifuged to remove insoluble matter, and an aliquot (100 µl) was injected into the HPLC system for analysis.

The majority of radioactivity (>74%) excreted in urine was recovered during 240 h post dose. Therefore, urine (approximately 50 ml) from each subject taken between 0 and 240 h post dose administration were pooled. Approximately 500 ml of urine from each subject was lyophilized; the residue reconstituted in 30 ml of water and passed over a conditioned C-18 SPE column (Supelco; Bellefonte, PA). The column was washed with water and the

radioactivity eluted with 5 ml of methanol. The overall recovery of radioactivity was about 82-88%. The methanolic eluate was concentrated under nitrogen, the residue reconstituted in 0.5 ml of methanol:water (1:1), and a 100 μl aliquot injected into the HPLC column for analysis.

Aliquots of fecal homogenates from 24-336 h for subject # 1-3 and from 24h-408 h for subject # 4 were pooled and the pooled samples were lyophilized. The dried residue was reconstituted in 120 ml of acetonitrile and stirred for 2 h using a magnetic stirrer. The mixture was centrifuged and the supernatant collected. The process of extraction was repeated three times with 120 ml of acetonitrile each time. The supernatants were collected and 250 μl aliquots were counted in a liquid scintillation counter. The overall recovery of radioactivity in feces was about 78-85%. The organic solvent was evaporated to dryness in a rotary evaporator, the residue reconstituted in 10 ml of methanol:water (1:1), and a 100 μl aliquot injected into the HPLC system for analysis.

In vitro Studies of Lasofoxifene Metabolism

Human Liver Microsomes and Recombinant Human CYPs Incubations

Human liver microsomes and recombinant CYP isoforms were reconstituted in 100 mM potassium phosphate butter (pH=7.4) prior to analysis. [^{14}C] Lasofoxifene (1 μM) was pre-incubated with microsomes (0.5 mg/ml protein microsomes) or recombinant enzyme (20 pmol/ml) for 3 min at 37 ^{o}C in a shaking water bath. The incubation was initiated with the addition of 100 μl cofactor (1.1 mM NADPH, 10 mmol $MgCl_2$) per 1 ml of incubation mixture. After 30 min, the incubations were terminated by the addition of 100 μl cold acetonitrile and ascorbic acid (50 μl of a 20 mM stock solution in water). Isoform specific inhibitors were used in conjunction with microsomal experiments to determine the isoform

responsible for the metabolism of lasofoxifene. The inhibition studies used CYP isoform-selective inhibitors (ketoconazole 1 μM, quinidine 1 μM, quercetin 40 μM, sulfaphenazole 40 μM, furafylline 40 μM, and ABT (100 μM). These were added to the microsomal incubations at the start of the incubation, except for furafylline and ABT, which were pre-incubated with NADPH for 5 min prior to the addition of lasofoxifene. Metabolites of lasofoxifene were extracted from the incubation mixtures by liquid:liquid extraction using two volumes of MTBE.

Human Liver S-9 and Cytosol Incubations

5-Hydroxy-lasofoxifene and 7-hydroxy-lasofoxifene (5 μM) were incubated at 37°C (30 min) in human cytosolic fractions with SAM (1 mM) to demonstrate these compounds as substrates for *in vitro* phase II reactions. Prior to use, sub-cellular fractions were thawed on ice and diluted 1:1 with 100 mM potassium phosphate pH 7.4. The substrates were dissolved in acetonitrile (1 mM) and added to the incubation mixture to a final substrate concentration of 1 μM. Incubations were stopped after 30 min by the addition of 100 μl cold acetonitrile and ascorbic acid (50 μl of a 20 mM stock solution in water).

Glucuronidation by Human liver and Intestinal Microsomes and Expressed UGTs

Prior to use, microsomes and UGTs were thawed on ice and reconstituted using 100 mM potassium phosphate pH 7.4. Lasofoxifene was dissolved in DMSO (5 mM) and added to the incubation mixture to a final substrate concentration of 25 μM. Samples were pre-incubated for 3 min (0.5 mg/ml protein) at 37°C in a shaking water bath. Incubations were initiated with the addition of 100 μl cofactor (alamethicin 25 μg/ml, UDPGA 3 mM, saccarolactone 25 μM, 8 mM $MgCl_2$) per 1 ml of incubation mixture. Incubations were stopped after 30 min by the addition of an equal volume of cold acetonitrile. Precipitated protein was

removed by centrifugation (14,000 rpm for 5 min). Supernatants were transferred to HPLC injection vials without further purification.

Microsomal and cytosolic incubations were passed over C-8 SPE for sample concentration. Spec® C-8, SPE cartridges (0.5 mg) were conditioned by the passing methanol (1 ml), followed by water (1 ml) over the column bed under slight vacuum. Samples were aspirated through the column under vacuum (<5mm Hg). The column beds were then washed with water (1 ml) and the samples were eluted with acetonitrile (2 x 250 µl aliquots). Following elution, ascorbic acid (50 µl of a 20 mM stock solution in water) was added and the samples were placed under nitrogen for concentration and reconstituted in 100 µl of mobile phase (10 mM ammonium formate/acetonitrile (70:30)

HPLC.

The HPLC system consisted of a HP-1050 solvent delivery system, a HP-1050 membrane degasser, an HP-1050 autoinjector (Hewlett Packard, Palo Alto, CA), a Thermo Separations spectromonitor 3200 UV (San Jose, CA) and a radioactive monitor (β-RAM, IN/US, Tampa, FL). Chromatography was performed on a Beckman Ultrasphere C-18 column (4.6 mm x 250 mm, 5 µm) with a mobile phase containing a mixture of 10 mM ammonium acetate (solvent A) and methanol (solvent B). The mobile phase was initially composed of solvent A/solvent B (80:20), held for 1 min, it was then linearly programmed to solvent A/solvent B (20:80) over 30 min followed by a short gradient to solvent A/solvent B (5:95) over 5 min. Chromatography was conducted under isocratic conditions for 10 min. The mobile phase composition was returned to the starting solvent mixture over 5 min. The system was allowed to equilibrate for approximately 15 min before making the next injection. A flow rate of 1.0 ml/min was used for all analyses.

For microsomal incubations, chromatography was performed on a Metachem Polaris C-18 column (4.6 mm x 250 mm, 3 µm). The mobile phase was initially composed of 0.05% formic acid/0.1% ammonium hydroxide in water containing 30% acetonitrile (solvent A) and acetonitrile (solvent B). The mobile phase was initially composed of solvent A/solvent B (90:10), and held for 12 min. The mobile phase composition was then linearly programmed to solvent A/solvent B (65:35) over 22 min and held at these conditions for 5 min. The mobile phase composition was returned to the starting solvent mixture over 2 min. The system was allowed to equilibrate for 10 min prior to the next injection. A flow rate of 1.0 ml/min was used for all analyses.

For cytosolic incubations, chromatography was performed on a Metasil AQ column (4.6 mm x 250 mm, 3 µm). The mobile phase was initially composed of 50:50 solvent A: solvent B. It was linearly ramped to 60:40 over 20 min and then to 20:80 in 5 min. It was held for 1 min and changed to 50:50 in 2 min. The system was allowed to equilibrate for 10 min prior to the next injection. A flow rate of 1.0 ml/min was maintained throughout the analyses.

For analysis of glucuronides, chromatography was performed on an Atlantis Polarity C-18 column (2.1 mm x 50 mm, 5 µm). The mobile phase was initially composed of 10 mM ammonium acetate pH 5.0 (Solvent A) and acetonitrile (Solvent B). The mobile phase was initially composed of 85:15 solvent A: solvent B. It was linearly ramped to 40:60 over 3 min and held for 2 min and changed to 85:15 in 1 min. The system was allowed to equilibrate for 3 min prior to the next injection. A flow rate of 1.0 ml/min was used for all analyses.

Quantitative Assessment of Metabolite Excretion

Quantification of metabolites in feces was carried out by measuring the radioactivity in the individual peaks that were separated on HPLC using β-RAM (IN/US, Tampa, FL). The β-

RAM provided and integrated printout in CPM and the percentage of the radiolabelled material, as well as the peak representation. The β-RAM was operated in the homogeneous liquid scintillation counting mode with the addition of 3 ml/min of Tru-Count scintillation cocktail (IN/US, Tampa, FL) to the effluent post-UV detection. The radiochromatograms of metabolites in plasma and urine were generated by collecting fractions at 0.33 min intervals and counting the fractions in a Packard #2500CA liquid scintillation counter.

In vitro metabolites were quantified by measuring the radioactivity in individually separated radioactive peaks using a LC-ARC system (Liquid Chromatography-Accurate Radioisotope Counting, AIM Research Company, Newark, DE). The LC-ARC was operated in the homogeneous liquid scintillation counting mode with the addition of 2.5 ml/min of Tru-Count scintillation cocktail (IN/US, Tampa, FL) to the effluent post-UV detection. The retention times of the radioactive peaks, where possible, were compared with those of synthetic standards.

LC/MS/MS

Identification of metabolites was performed on a Finnigan TSQ 7000 LC/MS/MS (Thermo Electron Corp., San Jose, CA) using pneumatically assisted electrospray. The effluent from the HPLC column was split and about 50 µl/min was introduced into the mass spectrometer. The remaining effluent was directed into the flow cell of the β-RAM. The β-RAM response was recorded in real time by the mass spectrometer data system, which provided simultaneous detection of radioactivity (RAD) and mass spectrometry data. The interface was operated at 4500 V, sheath pressure at 80 PSI, capillary temp at 200 ^{o}C and the mass spectrometer was operated in the positive mode. Collision induced dissociation (CID) studies were performed using argon gas at collision energy of 30-40 eV and at a pressure of 2 mTorr. Data output from the mass spectrometer was analyzed by Xcalibur (version 1.2, Thermo Electron Corp., San Jose, CA).

RESULTS

Excretion of [^{14}C]Lasofoxifene

Subjects were administered a single oral dose of an aqueous suspension containing 20 mg of [^{14}C]lasofoxifene. The majority of the radioactivity was excreted in the feces (Table 1), with a mean total of 66.1 ± 5.09% (SD) of the radioactivity at 576 h post dose administration. A mean total of 72.4 ± 5.86% (SD) of the radioactivity was excreted in the urine and feces at 576 h post dose administration. The cumulative mean (±SD) recovery of administered radioactivity in urine and feces is shown in fig. 2. Excretion of radioactivity via urine and feces continued for a considerable period of time, with 22.1% being excreted during the period 240–576 h post dose administration.

Pharmacokinetics

The mean (±SD) concentration-time profiles of lasofoxifene and total radioactivity in plasma of humans following oral administration [^{14}C]lasofoxifene are graphically depicted in fig. 3. Plasma concentrations for lasofoxifene and total radioactivity peaked at 4 and 12 h, respectively, after oral administration (Table 2). C_{max} values for unchanged lasofoxifene ranged from 11.5 to 20.3 ng/ml with a mean value of 17.1 ng/ml. C_{max} values for the total radioactivity ranged from 35.9 to 58.7 ng-equiv/ml with a mean value of 46.6 ng-equiv/ml. Mean $AUC_{(0-\infty)}$ values for the parent drug ranged from 2280 to 3110 ng.h/ml with a mean value of 2620 ng.h/ml. Mean $AUC_{(0-168)}$ for total radioactivity was 3290 ng-equiv.h/ml. The mean terminal phase ($T_{1/2}$) of lasofoxifene was estimated as 153 ± 28 h. The C_{max} and AUC values of unchanged lasofoxifene were lower than those determined for radioactivity, indicating presence of circulating metabolites in plasma (Table 2). The AUC_{0-168} ratios of lasofoxifene to total radioactivity suggested that approximately 54% of the total circulating radioactivity was attributable to the metabolites (Table 2).

Identification of Metabolites

Urine.

HPLC analysis of pooled urine collected 0–240 h post dose administration identified five metabolites of lasofoxifene: the mono-hydroxylated (M21) and methylated catechol (M17) metabolites; and a glucuronide conjugate (M7), with its mono- and di-hydroxy derivatives (M9 and M23/M24) (Fig. 4). Identification of these metabolites was performed by LC/MS/MS using multiple reaction monitoring (MRM) and by comparison of their HPLC retention times with the metabolites identified in rat and monkey (Johnson and Prakash, 1998). The mean percentages of each of these metabolites in urine in relation to the administered dose of lasofoxifene are shown in Table 3. Unchanged lasofoxifene accounted for only 1.77% of the dose in urine collected from 0–240 h post dose, and the glucuronide conjugate M7 was the most abundant metabolite (1.47%).

Feces.

A total of six metabolites of lasofoxifene were found in pooled samples of feces collected 24–336 h (three subjects) and 24–408 h (one subject) post dose administration: the catechol intermediate (M21), its sulfate conjugate M10, and its methylated conjugate M17; the sulfate conjugate of lasofoxifene (M11); M15, the product of the oxidation of the pyrrolidine moiety; and M12, which resulted from the aromatic hydroxylation of lasofoxifene (Fig. 5). Table 3 shows the mean percentages of each of these metabolites in feces in relation to the administered dose of lasofoxifene. The greatest proportion of radioactivity in feces was attributable to unchanged lasofoxifene (21.8%), with similar proportions for each of the metabolites (5.10–8.42%).

Plasma.

Plasma samples pooled at 24 and 48 h post dose for each subject contained lasofoxifene and three metabolites as demonstrated by HPLC analysis. The three metabolites detected were the glucuronide conjugate of lasofoxifene (M7), its hydroxylated metabolite (M9), and a methylated catechol (M17). These metabolites were identified by LC/MS/MS using multiple reaction monitoring (MRM) and by comparison of their HPLC retention times with the metabolites identified in rat and monkey (Fig. 6). Table 3 shows the percentage of each of these metabolites and lasofoxifene circulating in plasma for each subject. Approximately one-third of the radioactivity in plasma was attributable to unchanged lasofoxifene, and the most common metabolite was the glucuronide conjugate M7 (21.8%).

In Vitro Metabolism of Lasofoxifene

Human Liver Microsomes

HPLC-selected ion chromatograms of metabolites of lasofoxifene in human liver microsomes are shown in fig. 7. Lasofoxifene has a very low turnover in HL-mix-13 (~6%); the two metabolites formed were 5-hydroxy- (M21A; 5.11%) and 7-hydroxy-lasofoxifene (M21B;1.19%). These metabolites were not detected in the incubations devoid of NADPH, suggesting that the metabolism of lasofoxifene was mediated by CYP isoforms.

Recombinant CYP Isoforms

The ability of specific CYP isoforms to metabolize lasofoxifene was determined using recombinant CYP1A1, CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5. Incubations of lasofoxifene with CYP 3A5 and 2D6 formed 7-hydroxy metabolite (M21B) as the major product (44.7% and 58.0%, respectively). It was also observed in the incubations with CYP 2C19 and 1A1, but at a much lesser extent (1.1% and 5.8%, respectively). 5-Hydroxy-lasofoxifene (M21A) was formed primarily by CYP3A4 (57.6%) and CYP3A5 (11.1%). These oxidative metabolites were not detected in incubations

containing recombinant CYP1A2, CYP2C8, CYP2C9 or CYP2E1. As the enzyme activities of the recombinant CYPs were selected to reflect activities typical of a human liver microsomal preparation, it can be concluded that these latter isoforms do not contribute significantly to lasofoxifene metabolism.

Inhibition studies

Inhibition of metabolite formation by each inhibitor of specific CYP (isoform) is summarized in table 4. Ketoconazole inhibited the formation of both 5-hydroxy-lasofoxifene M21A (83%) and 7-hydroxy-lasofoxifene M21B (100%). While quinidine inhibited 7-hydroxy- and 5-hydroxy metabolites formation, by 61.3 and 10.2%, respectively. ABT showed 100% inhibition of these two metabolites. Sulfaphenazole (37.8%), furafylline (45.4%) and quercetin (60.5%) showed moderate inhibition for the formation of 7-hydroxy-lasofoxifene. The formation of 5-hydroxy-lasofoxifene was also inhibited by sulfaphenazole (3.13%), furafylline (14.9%) and quercetin (22.5%). Addition of the isoform specific inhibitors ketoconazole to CYP3A4 and 3A5 or quinidine to CYP2D6 isoform incubations showed 100% inhibition of metabolite production.

Cytosolic Fraction

HPLC-selected ion chromatograms of metabolites of 5-hydroxy- and 7-hydroxy-lasofoxifene with human liver cytosols in the presence of SAM are shown in fig. 8. 5-hydroxy- and 7-hydroxy-lasofoxifene each yielded a mixture of two mono-methylated regioisomers. This data suggests that *in-vivo*, a mixture of 5-hydroxy- and 7-hydroxy-lasofoxifene may form 4 regioisomers of the mono methylated catechol metabolite (M17).

Lasofoxifene Glucuronidation by Human liver and Intestinal Microsomes and Expressed UGTs

Human liver and intestinal microsomes and various UGT isoforms (UGT 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17) were tested for their ability to catalyze lasofoxifene glucuronidation. Human liver and intestinal microsomes produced a major glucuronide metabolite M7 that was identified *in vivo* in humans. From the 12 UGTs tested, six (1A1, 1A3, 1A6, 1A8, 1A9 and 1A10) were able to catalyze the formation of the lasofoxifene glucuronide (M7).

DISCUSSION

The aims of this study were to characterize the metabolism and excretion of lasofoxifene in humans and to identify the enzymes (CYPs and UGTs) involved in its metabolism. A single 20-mg dose of [^{14}C]lasofoxifene was well tolerated by all four subjects in this study. There were no serious adverse events or withdrawals due to adverse events.

Recovery of radioactive dose in human was 72.4% on average over a period of 24 days, and ranged from 64.3% to 78.0% in the four subjects. Small amounts of radioactivity were excreted up through the last day of collection with 0.6% to 1.5% recovered in feces on Day 24. From days 11 to 24, 20.3% of the dose was excreted in feces and 1.8% was excreted in urine. Examination of the rate of excretion of radioactivity suggests that if the study could have been continued beyond 24 days, recovery would have increased. The majority (66.1%) of radioactive dose was excreted in the feces, which is likely the consequence of biliary excretion of metabolites. In the bile duct cannulated rats, 83.0% of the dose was excreted in the bile over 48 h of which 56% was identified as glucuronide conjugate (manuscript in preparation). Therefore, the unchanged drug in feces of humans is thought to be due to

hydrolysis of lasofoxifene glucuronide by gut microflora rather than incomplete absorption. The excretion of lasofoxifene radioactivity primarily via the feces was similar to that of raloxifene, a SERM already used for the treatment and prevention of osteoporosis in postmenopausal women (Hochner-Celnikier et al., 1999; Snyder et. al., 2000; Heringa et al., 2003; Morello et al., 2003). In humans, only 6.4% of the dose was excreted in urine, with <2% as unchanged lasofoxifene. This indicates renal elimination is not an important clearance mechanism for lasofoxifene and there should be little impact on lasofoxifene pharmacokinetics in patients with impaired renal function.

Lasofoxifene was readily metabolized in humans, with a total of nine metabolites being identified by LC-MS/MS using MRM technique (Prakash et. al. 2007). A total of six metabolites were found in feces, five in urine and three in plasma. All of these metabolites have been identified previously in rats and/or monkeys (Johnson and Prakash, 1998). In excreta, identified metabolites (including lasofoxifene) ranged from 94% of the recovered dose in humans. Based on the structures of the metabolites, six major metabolic pathways (both Phase I oxidation and phase II conjugation with glucuronic acid or sulfate) have been shown to be responsible for the metabolism of lasofoxifene (Fig. 9). Lasofoxifene was a major component of the radiolabel eliminated in the feces, but this is thought to be the result of hydrolysis of lasofoxifene glucuronide by gut microflora (Scheline, 1973). If the assumption is made that lasofoxifene in feces represents biliary excretion of lasofoxifene glucuronide, then it can be estimated that conjugation accounts for up to 50% of the direct metabolism of lasofoxifene in humans.

Unlike raloxifene (Hochner-Celnikier et al., 1999; Snyder et al., 2000), lasofoxifene also underwent phase I reactions in addition to conjugation. For example, hydroxylation of the

tetraline ring produced the catechol intermediate M21, which was either conjugated via sulfation to form M10 or underwent O-methylation possibly by COMT, an enzyme present in liver, red blood cells and other extrahepatic tissue (Lipsett et al., 1983; Emons et al., 1987) to form M17. The catechol intermediate M21 was also hydroxlylated and conjugated with glucuronic acid to form M23/M24. However, in feces, conjugated metabolites were identified as the aglycones, possibly due to hydrolysis of conjugates by gut microflora (Scheline, 1973). A significant portion of the administered radioactivity was excreted as unchanged lasofoxifene. It could be envisioned that this is likely to be the result of hydrolysis of lasofoxifene glucuronide by gut microflora and not unabsorbed drug (Scheline, 1973). Metabolite M15, only found in feces, was formed by the oxidation of the pyrrolidine moiety. Metabolite M12 was formed by the hydroxylation on the 5-phenyl group of lasofoxifene.

In addition to lasofoxifene, three metabolites were identified in the pooled 24-48 hour plasma samples. Lasofoxifene, its glucuronide (M7) and the methylated catechol metabolite (M17) were identified the major circulating metabolites in humans. The hydroxylation at the tetraline ring could form two regioisomers of catechol intermediate (5-hdroxy- (M21A) and 7-hydroxy- (M21B) lasofoxifene). COMT could then generate four isomeric monomethyl ethers from these catechol intermediates, i.e. 5-methoxy-, 5-hydroxy-6-O-methyl, 7-methoxy, and 7-hydroxy-6-O-methyl-lasofoxifene. Due to low abundance of these metabolites in humans, the exact position of methylation could not be determined. Analysis of plasma from postmenopausal women confirmed that the lasofoxifene metabolites seen in male plasma were also present in the plasma of postmenopausal women (not shown).

Oxidative metabolites, most likely the result of P450-dependedent activity and therefore, *in vitro* metabolism of lasofoxifene was studied in human liver microsomes, and recombinant CYP isoforms to determine the CYP isoform(s) responsible for its oxidative metabolism. Incubation of lasofoxifene with human liver microsomes resulted in the formation of only two catechol metabolites, 5-hydroxy- (M21A)and 7-hydroxy-lasofoxifene (M21B). Further results from experiments with recombinant CYP isoforms and inhibition studies suggested that 5-hydroxy-lasofoxifene (M21A) was formed primarily by CYP3A4 and CYP3A5, while 7-hydroxy-lasofoxifene (M21B) was formed by CYP2D6 and CYP3A5 and to a much lesser extent by CYP2C19 (1.1%). The *in vitro* metabolism studies of these catechol metabolites with human S-9 fraction in the presence of SAM formed a mixture of two mono-methylated regioisomers of each standard compound. This data suggests that metabolite M17 identified *in vivo* in humans may represent a mixture of four regioisomers of the mono methylated catechols.

Since glucuronidation also plays an important role in the metabolism of lasofoxifene and therefore, *in vitro* studies were conducted in human liver and intestinal microsomes and expressed UGTs. The results from these suggested that glucuronidation of lasofoxifene is catalyzed by UGTs that are expressed in both the liver (1A1, 1A3, 1A6 and 1A9), and the intestine (1A8 and 1A10). The relative contribution of various UGTs is now under investigation and will be reported separately.

The major circulating metabolites M17 (four regioisomers) and M7 were synthesized and tested *in vitro* for the ability to bind to either ERα or ERβ and were found to have significantly lower affinity than lasofoxifene (not shown). Based on the *in vitro* findings and

the observed circulating concentrations in human subjects, it is not likely that any of these metabolites contribute to the pharmacologic activity of lasofoxifene.

Although lasofoxifene is extensively hydroxylated in humans but the non-conjugated catechol metabolite was not detected in systemic circulation. It is probably due to rapid conjugative metabolism (O-methylation and subsequent glucuronidation or sulfation) followed by urinary excretion. It is well established that the catechols are metabolized to highly reactive *o*-quinone by monooxygenase or peroxidase enzymes, metal ions or molecular oxygen (Monks et al., 1992; Bolton et. al., 2000). However, unlike raloxifene, no glutathione conjugate of lasofoxifene was detected either in humans or preclinical species, suggesting that the formation of catechol is rate limited (Chen et al., 2002).

This study has clearly shown that the most important route of elimination of lasofoxifene was via metabolism and excretion in the feces. The study presented evidence that both conjugation and oxidation reactions were involved in the metabolism of lasofoxifene in humans. The results from *in vitro* experiments with recombinant isoforms and CYP-isoform selective inhibitors suggested that the oxidative metabolism of lasofoxifene is catalyzed primarily by CYP3A4/3A5 and CYP2D6 and its glucuronidation is catalyzed by UGTs that are expressed in both the liver (1A1, 1A3, 1A6 and 1A9), and the intestine (1A3,1A8 and 1A10) (Tukey and Strassburg, 2000). Since oxidation accounts for about half of total lasofoxifene clearance and this pathway is further divided among multiple CYP isozymes, inhibition of a single enzyme will not have a clinically significant impact on the pharmacokinetics of lasofoxifene. Phase 2 clinical studies showed only modest increase in both the AUC and C_{max} of lasofoxifene with concomitant administration of either ketoconazole, a potent inhibitor of CYP3A4 (Ouellet et. al. 2006) or paroxetine, a potent

inhibitor of CYP2D6 (Ouellet et. al. 2006). Considering the large safety margin for lasofoxifene, no dosage adjustment is needed when coadministered with either CYP3A or CYP 2D6 inhibitors (Ouellet et. al. 2006). However, as a result of these data, there is the potential for drug–drug interactions with any co-administered drug that is also eliminated by the same metabolic pathways, particularly those involving phase I oxidation by microsomal CYP enzymes.

In conclusion, this metabolism and excretion study in healthy subjects has demonstrated that lasofoxifene, a new generation SERM, is primarily eliminated via metabolism and fecal excretion comparable with that of other SERMs, such as tamoxifen, toremifene and raloxifene (Morello et. al., 2003). The primary clearance mechanisms for lasofoxifene in humans appear to be due to direct conjugation and P450-mediated oxidation, each accounting for half of the metabolism. CYP3A4/5 and CYP2D6 are the primary CYP isoforms involved in the oxidative metabolism and several UGTs of 1A family are involved in the glucuronidation of lasofoxifene.

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Footnotes:

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Figure Legends:

FIG. 1. The structure of lasofoxifene (* denotes the positions of ^{14}C-label)

Fig. 2. Cumulative mean (± SD) recovery of administered radioactivity in urine and feces of humans (*n*=4) following a single 20-mg oral dose of [^{14}C]lasofoxifene

FIG. 3. Mean (±SD) plasma concentration–time curves for lasofoxifene and total radioactivity in humans (*n*=4) following oral administration of a single 20-mg dose of [^{14}C]lasofoxifene

FIG. 4. Representative HPLC-radiochromatogram of urinary metabolites of lasofoxifene in humans following oral administration of a single 20-mg dose of [^{14}C]lasofoxifene

FIG. 5. Representative HPLC-radiochromatogram of fecal metabolites of lasofoxifene in humans following oral administration of a single 20-mg dose of [^{14}C]lasofoxifene

FIG. 6 SRM profiles of circulating metabolites of lasofoxifene in humans

FIG. 7. Extracted ion chromatograms of laxofoxifene metabolites produced by human liver microsomes

FIG. 8. Selected ion chromatograms of metabolites of 5-hydroxy- (A) and 7-hydroxy-lasofoxifene (B) in human liver cytosol in the presence of SAM

FIG. 9. Proposed metabolic pathways of lasofoxifene in humans

TABLE 1

Percentage of radioactivity excreted in urine and feces of humans (*n*=4) following oral administration of a single 20-mg dose of [^{14}C]lasofoxifene

	Percent Dose		
Subject #	Urine	Feces	Total
1	7.2	70.8	78.0
2	5.39	58.9	64.2
3	7.02	67.6	74.6
4	5.94	67	72.9
Mean	6.39	66.1	72.4
SD	0.87	5.09	5.86

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TABLE 2

Pharmacokinetic parameters for lasofoxifene and total radioactivity in humans (*n*=4) following a single 20-mg oral dose of [^{14}C]lasofoxifene

		Pharmacokinetic parameter				
Analyte	Subject number	T_{max} (h)	C_{max}[a] (ng/ml)	AUC(0–168)[a] (ng·h/ml)	AUC(0–∞)[a,b] (ng·h/ml)	$t_{½}$[b] (h)
Lasofoxifene	1	8	19.1	1620	2670	134
	2	24	11.5	1220	2400	178
	3	8	17.3	1470	2280	124
	4	8	20.3	1690	3110	175
	Mean	12	17.1	1500	2620	153
	SD	7	4.0	208	368	28
	CV%	58	23	14	14	18
Total	1	4	41.3	3320	–	–
Radioactivity	2	4	35.9	2580	–	–
	3	4	50.5	3810	–	–
	4	4	58.7	3440	–	–
	Mean	4	46.6	3290	–	–
	SD	0	8.7	447	–	–
	CV%	0	19	14	–	–

[a]C_{max} and AUC values for total radioactivity are expressed as ng equiv/ml and ng equiv·h/ml, respectively; [b]Total radioactivity could not be measured past 168 h and consequently $t_{½}$ and

AUC(0-∞) values were not estimated; SD, standard deviation; CV%, percentage coefficient of variation.

TABLE 3

Mean percentage of metabolites of lasofoxifene in plasma, urine and feces in humans (*n*=4) following oral administration of a single 20-mg dose of [^{14}C]lasofoxifene

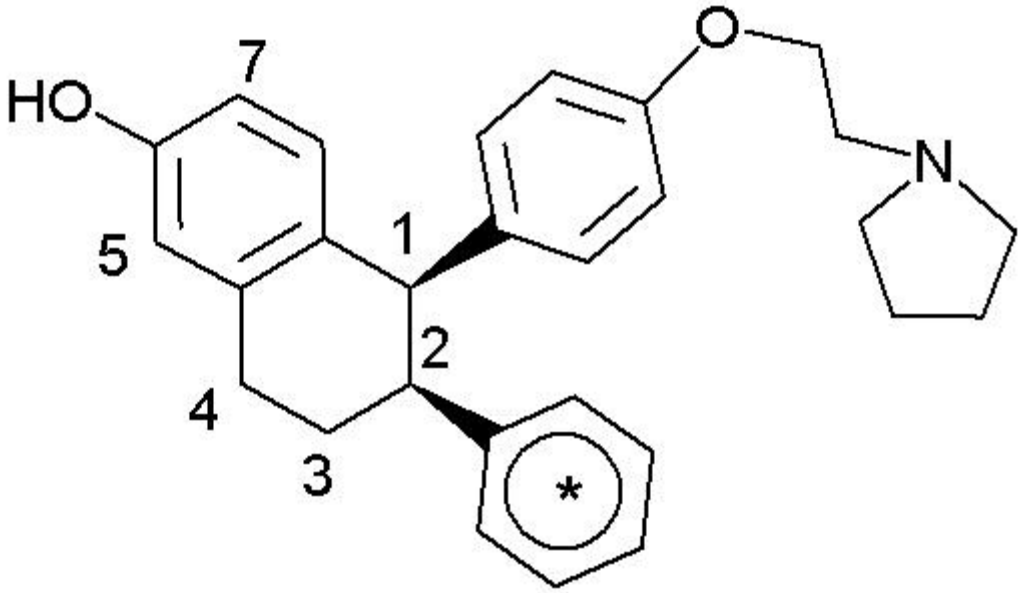
Metabolite	Percentage of radioactivity in plasma* Mean (SD)	Percentage of dose in urine†[a] Mean (SD)	Percentage of dose in feces†[b] Mean (SD)
M7	21.8 (9.2)	1.47 (0.68)	–
M9	6.4 (1.1)	0.46 (0.09)	–
M10	–	–	7.79 (1.66)
M11	–	–	8.42 (3.00)
M12	–	–	8.05 (2.60)
M15	–	–	7.37 (1.88)
M17	6.2 (2.5)	<0.1 (0.00)	6.02 (0.97)
M21	–	–	5.10 (1.84)
M23/M24	–	0.28 (0.17)	
Lasofoxifene	33.6 (6.3)	1.77 (1.12)	21.8 (7.96)

*Sample taken from pooled plasma at 24 and 48 h post dose administration; †Percentage of metabolite in relation to the administered dose of [^{14}C] lasofoxifene; [a]Sample taken from pooled urine collected 0–240 h post dose administration; [b]Sample taken from pooled feces collected 24–336 h (three subjects) and 24–408 h (one subject) post dose administration; SD, standard deviation.

TABLE 4

Effects of isoform-selective inhibitors of human cytochrome P450 isoforms on the metabolism of lasofoxifene by human liver microsomes

Inhibitor	Conc (μM)	Inhibited CYP isoforms	Percentage inhibition 7-hydroxy-lasofoxifene	Percentage inhibition 5-hydroxy-lasofoxifene
Control	–		0.0	0.0
Furafylline	40	CYP1A1/1A2	45.4	14.9
Quercetin	40	CYP2C8	60.5	22.5
Sulfaphenazole	40	CYP2C9/2C19	37.8	3.10
Quinidine	1	CYP2D6	61.3	10.2
Ketoconazole	1	CYP3A4	100	82.6
ABT	100	CYP	100	100



*Denotes the position of ^{14}C-label

Fig. 1

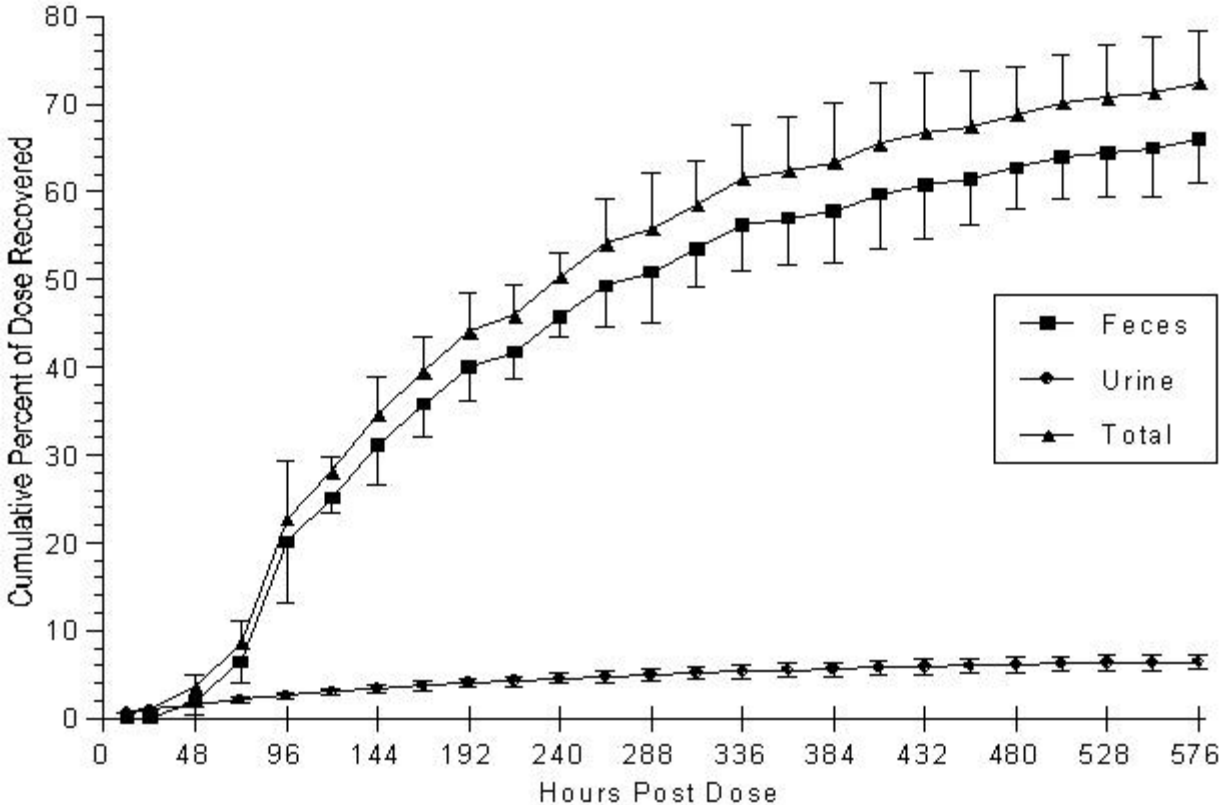

Cumulative Percent of Dose Recovered
80
70
60
50
40
30
20
10
0
0
48
96
144
192
240
288
336
384
432
480
528
576
Hours Post Dose
Feces
Urine
Total

Fig 2.

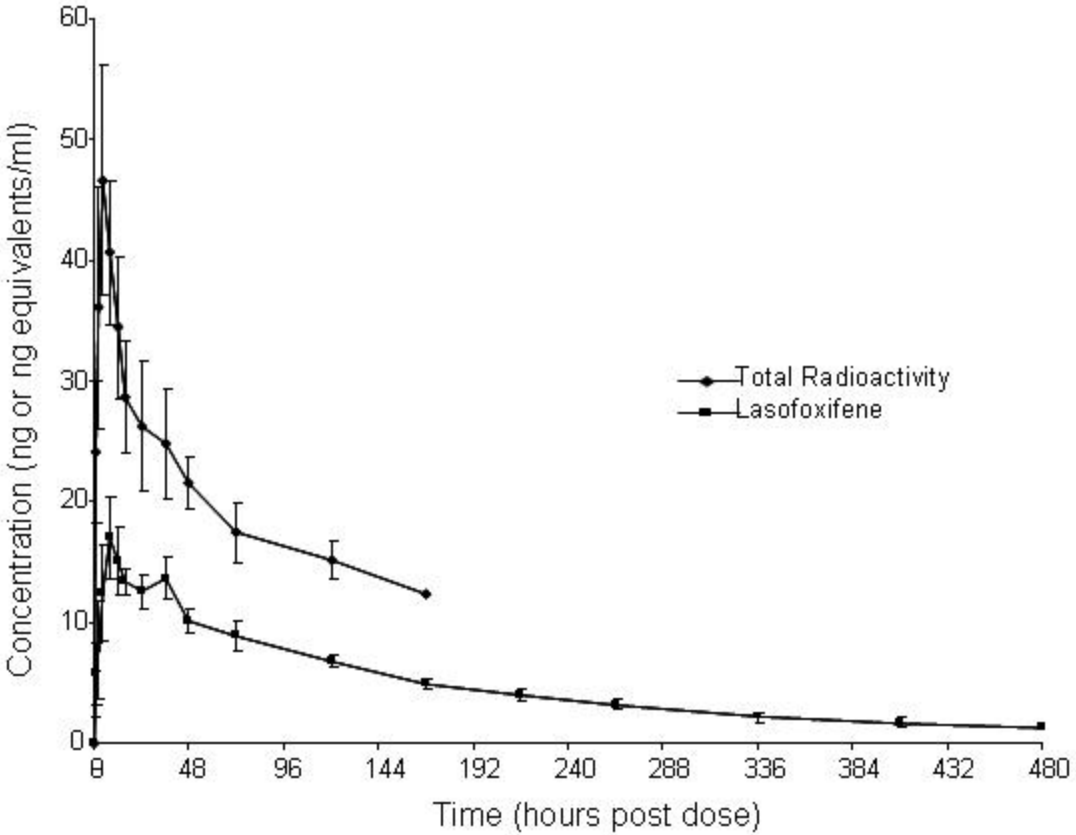

Concentration (ng or ng equivalents/ml)
60
50
40
30
20
10
0
0
48
96
144
192
240
288
336
384
432
480
Time (hours post dose)
Total Radioactivity
Lasofoxifene

Fig. 3

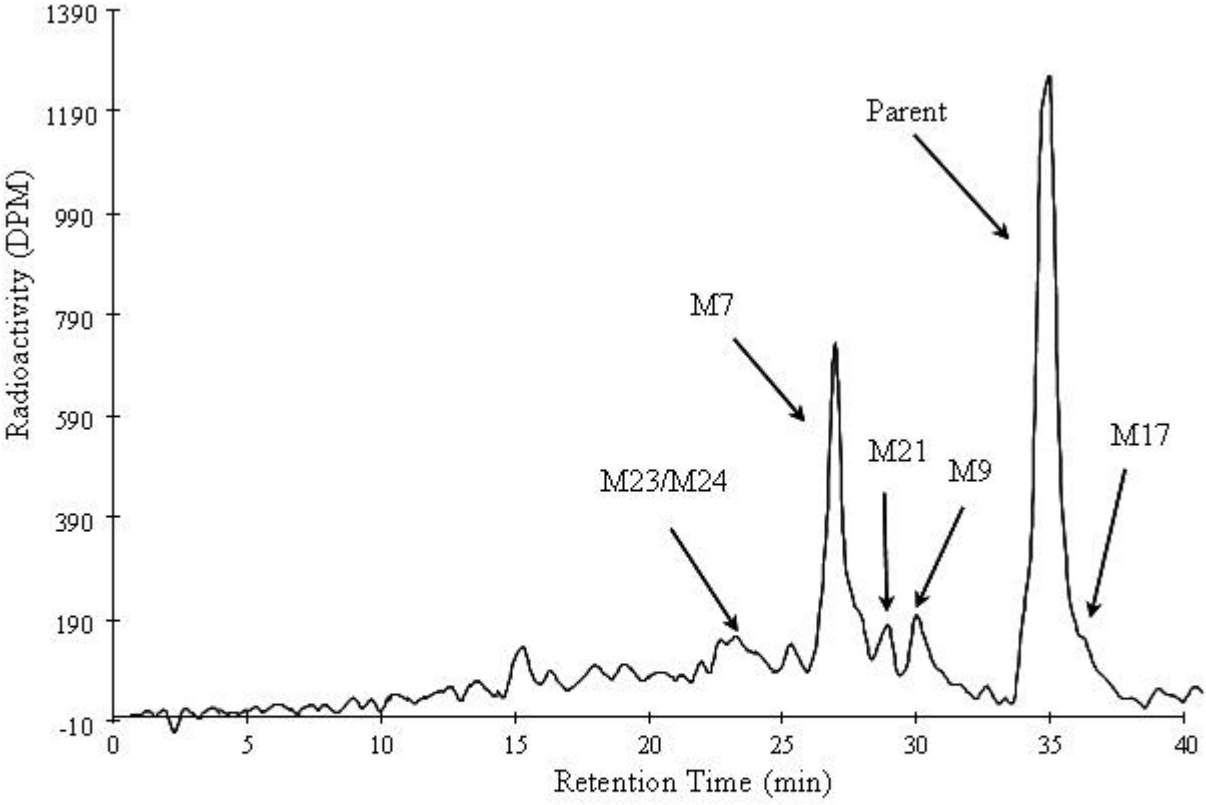

1390
1190
990
790
590
390
190
-10
Radioactivity (DPM)
0
5
10
15
20
25
30
35
40
Retention Time (min)
Parent
M7
M23/M24
M21
M9
M17

Fig. 4

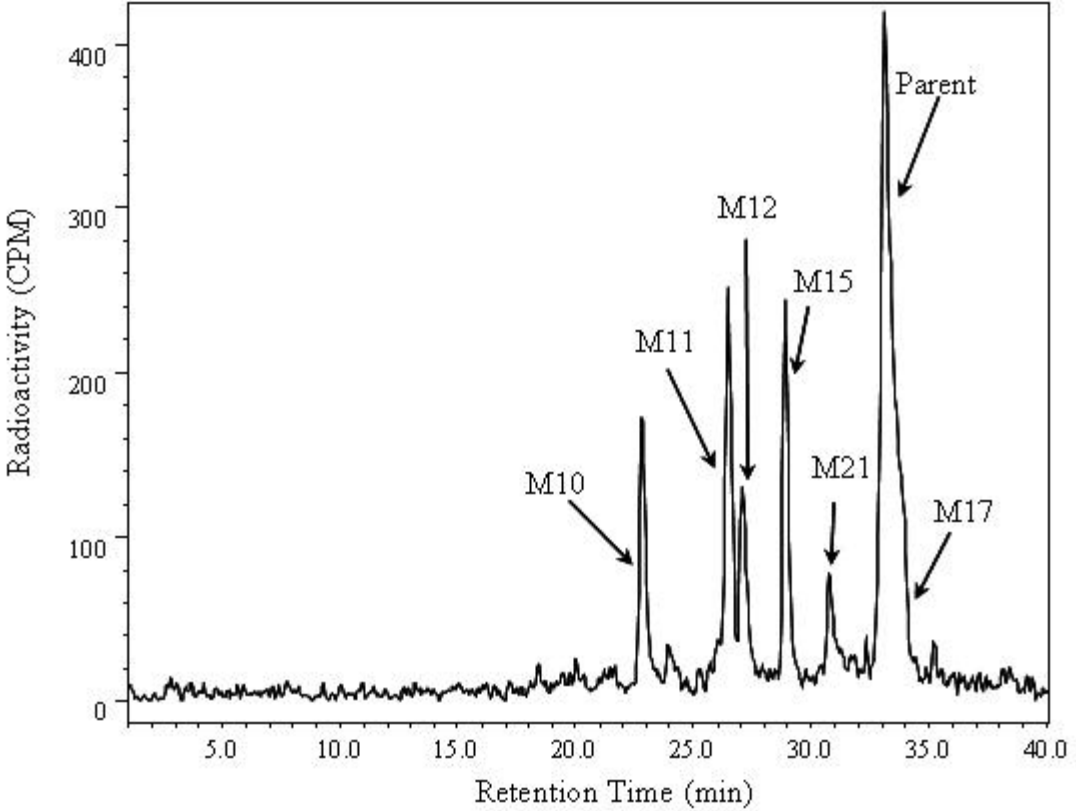

Radioactivity (CPM)
400
300
200
100
0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
Retention Time (min)
Parent
M12
M15
M11
M10
M21
M17

Fig 5

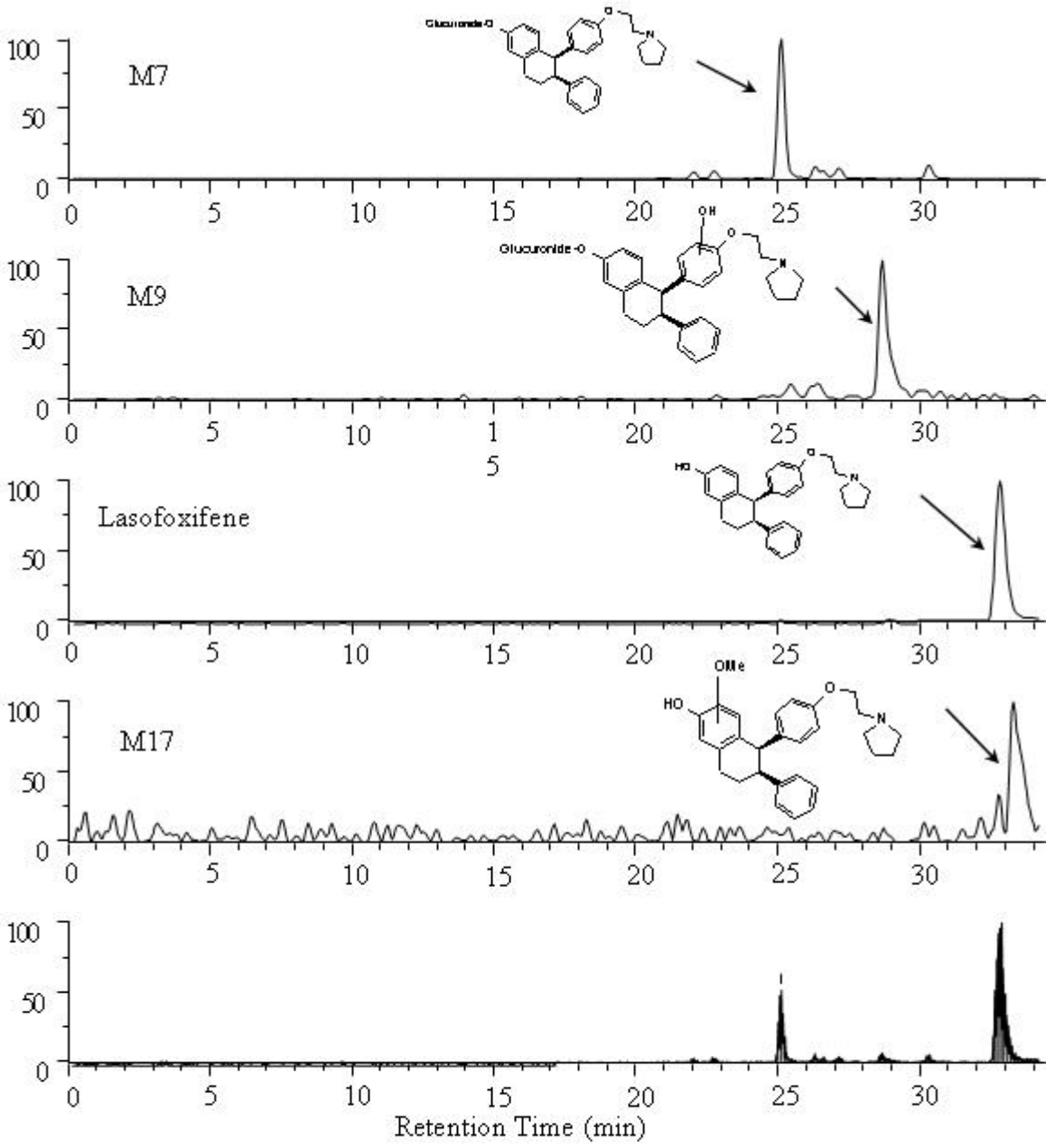

M7
M9
Lasofoxifene
M17
100
50
0
0
5
10
15
20
25
30
Retention Time (min)

Fig 6.

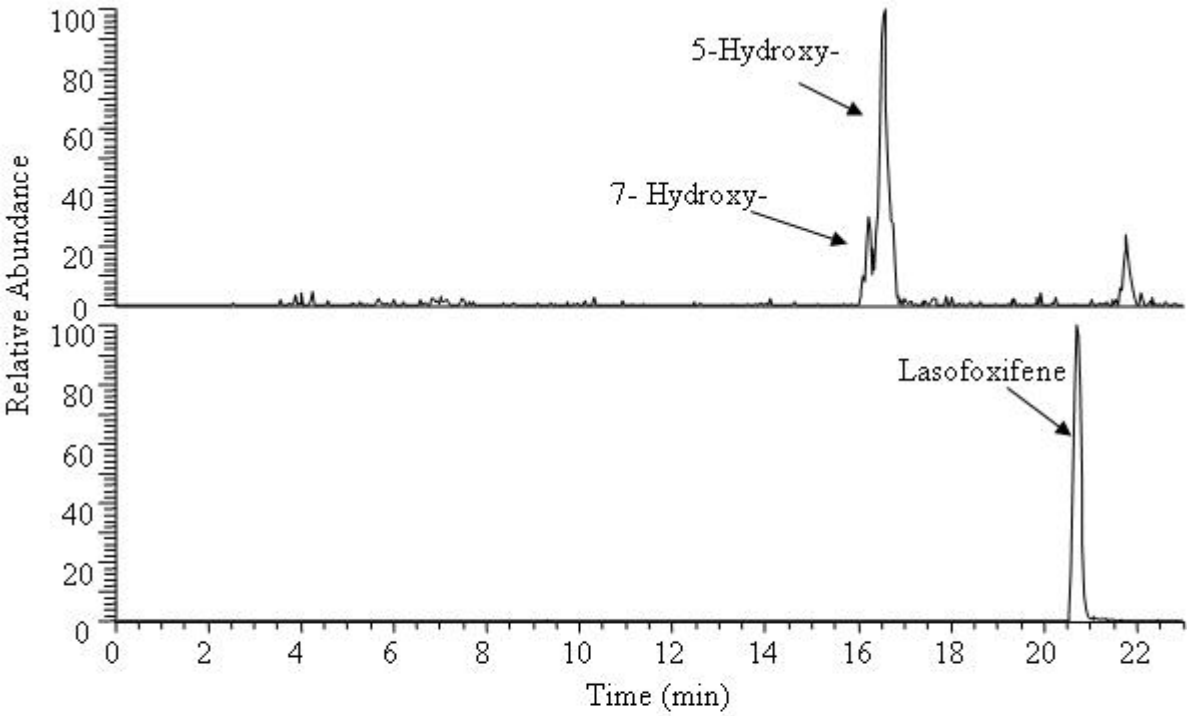

5-Hydroxy-
7- Hydroxy-
Lasofoxifene
Relative Abundance
Time (min)
0
20
40
60
80
100
0
2
4
6
8
10
12
14
16
18
20
22

Fig.7

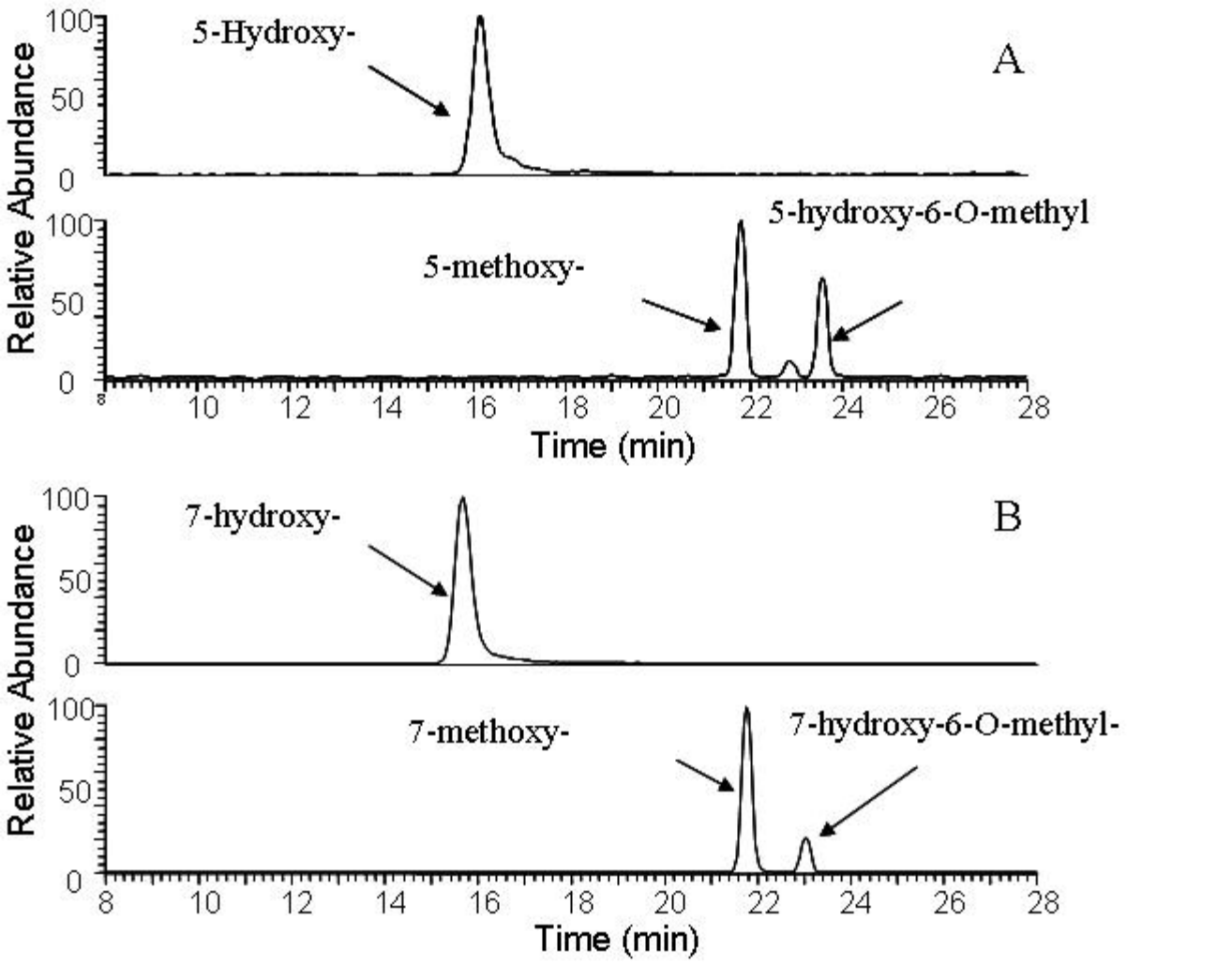
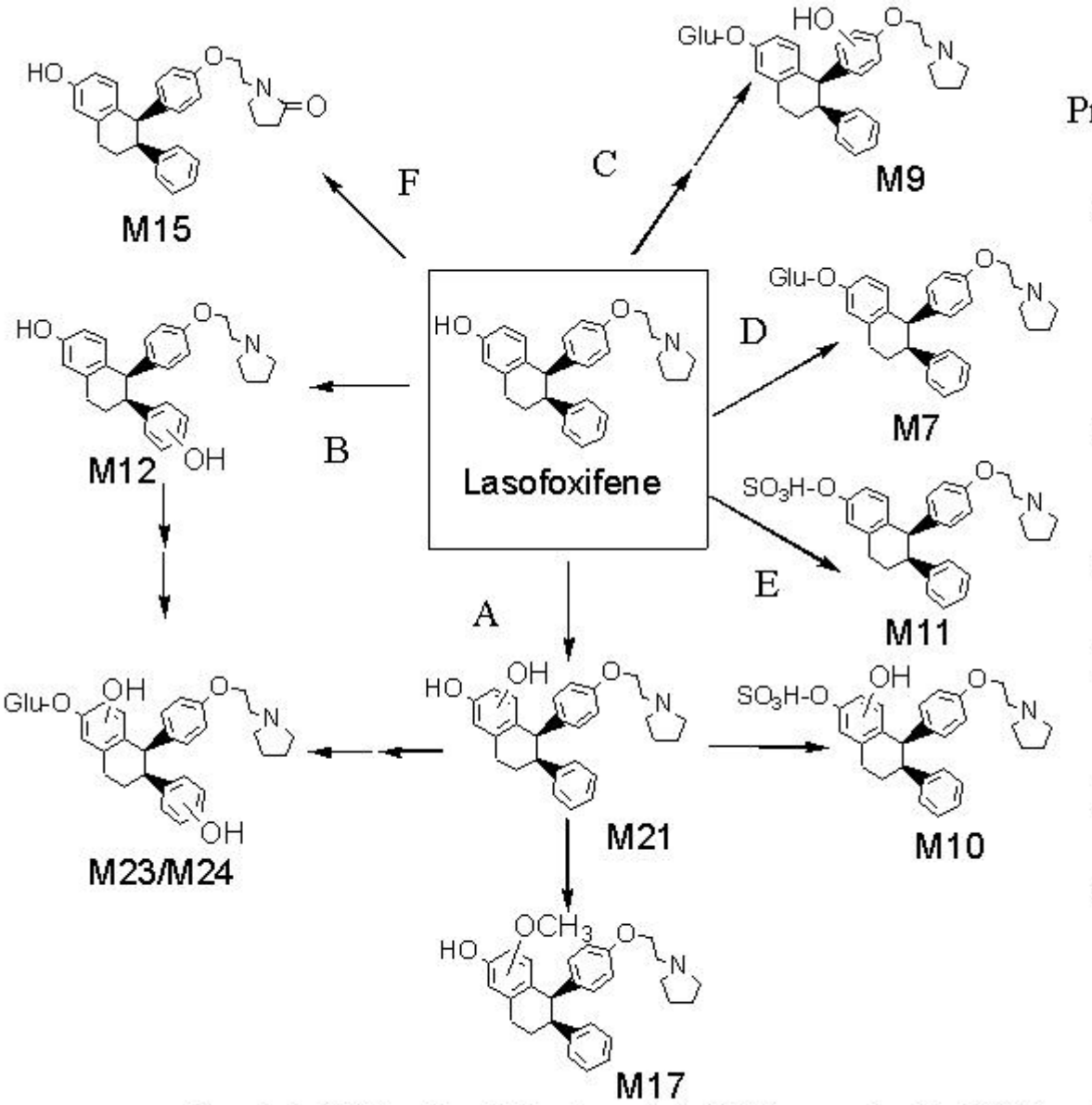

5-Hydroxy-
A
5-hydroxy-6-O-methyl
5-methoxy-
Relative Abundance
100
50
0
10
12
14
16
18
20
22
24
26
28
Time (min)
7-hydroxy-
B
7-methoxy-
7-hydroxy-6-O-methyl-
8

Fig 8



Primary Clearance Mechanisms

A. Aromatic hydroxylation
- M21 (5-OH, 7-OH catechols)
- M17 (circulating metabolite)
- M23/M24
- M10

B. Aromatic hydroxylation 2
- M12
- M23/M24

C. Aromatic hydroxylation 3
- M9 (circulating metabolite)

D. O-Glucuronidation
- M7 (circulating metabolite)
- Parent drug in feces

E. Sulfation
- M11

F. Pyrrolidine ring oxidation
- M15

Catechols (M21) and methylated catechols (M17) unresolved in ADME study but resolved in subsequent *in vitro* studies

FIG.9