METABOLISM, DISPOSITION, EXCRETION, AND PHARMACOKINETICS OF LEVORMELOXIFENE, A SELECTIVE ESTROGEN RECEPTOR MODULATOR, IN THE RAT

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ABSTRACT:
The tissue distribution, pharmacokinetics, metabolism, and excretion of the selective estrogen receptor modulator levormeloxifene have been investigated after oral administration of [14C]-levormeloxifene to male and female Sprague-Dawley rats. The quantitative distribution of radiolabeled levormeloxifene and/or metabolites was confirmed by whole body autoradiography. Levormeloxifene was absorbed from the gastrointestinal tract and was widely distributed into tissues, with peak radioactive concentrations generally being observed 4 h after administration in the intestine, liver, lung, kidney, spleen, pancreas, adrenals, and ovary (females). Fecal elimination was the major excretion route of radioactivity. In a separate pharmacokinetic study, plasma $C_{max}$ was generally observed 6 h after dose administration and the half-life of elimination was long (24 h) and a doubling in dose resulted in an approximate doubling in exposure. The majority of the drug was excreted as norlevormeloxifene; the 7-desmethyl metabolite of levormeloxifene, via the formation of phase II metabolites (glucuronides) and excretion into the bile. Unchanged drug was also excreted, mainly from 0 to 24 h, and accounted for about 6 to 12% of the dose. Together these two components accounted for approximately 50% of the radioactivity excreted. Additional metabolites isolated and identified by liquid chromatography-tandem mass spectrometry, and accounting for 1 to 5% of the excreted radioactivity in rat feces during the first 24 h, included two monohydroxylevormeloxifene species, a pyrrolidinone ring-opened metabolite of levormeloxifene, and desmethylnorlevormeloxifene.

Estrogen replacement therapy (ERT)¹ has been shown to be effective in both preventing postmenopausal osteoporosis and in reducing the risk of cardiovascular disease (Witt and Lousberg, 1997). However, without the concomitant administration of progesterone supplements, ERT has been associated with an increased stimulation of the endometrium, causing hyperplasia and risk of cancer. Thus, there is an interest in developing oral drugs that possess the beneficial effects of ERT, such as osteoporosis prevention, but do not have any detrimental effect on the uterus.

One such candidate, levormeloxifene ((-)-3,4-trans-7-methoxy-2,2-dimethyl-3-phenyl-4-[4-(2-pyrrolidin-1-yl)ethoxy]phenyl]chromane, hydrogenfumarate), is a selective estrogen receptor modulator, with low intrinsic estrogenicity that has been shown to prevent osteopenia in the ovariectomized rat model of human osteoporosis (Bain et al., 1997), and to prevent aortic cholesterol accumulation in the ovariectomized rabbit model (Holm et al., 1997). In addition, levormeloxifene has an apparently unique estrogenic effect on the uterus of ovariectomized animals whereby uterine weight is increased with no evidence of epithelial proliferation or glandular stimulation (Bain et al., 1997; Korsgaard et al., 1997).

Levormeloxifene was selected as a development candidate for the prevention and treatment of postmenopausal osteoporosis and it has been postulated that it could provide an alternative to current ERTs because no epithelial or glandular proliferation in the uterus or associated tissue has been observed in animal species or postmenopausal human volunteers treated with levormeloxifene. The drug, whose structure is shown in Fig. 1, is the $l$-enantiomer of ormeloxifene, and the following preclinical studies were performed to characterize the

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¹ Abbreviations used are: ERT, estrogen replacement therapy; LC-MS-MS, liquid chromatography-tandem mass spectrometry; WBA, whole body autoradiography; AUC, area under the plasma concentration versus time curve; APCI, atmospheric pressure chemical ionization.

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* indicates position of radiolabel.

FIG. 1. The structure of levormeloxifene.
disposition and excretion of this new selective estrogen receptor modulator, because there were very little preclinical metabolic data available for the new antiestrogens that are currently in clinical trials (Lindstrom et al., 1984; Tanaka et al., 1994; O’Donnell et al., 1998). However, the development of this compound has recently been stopped due to a number of adverse events being reported during phase III clinical trials, but new indications are currently being pursued as preclinical testing is near completion. It is anticipated that data generated within drug metabolism may contribute to the overall evaluation of new indications.

Materials and Methods

Chemicals. [14C]-Radiolabeled levormeloxifene (3,4-trans-7-methoxy-2,2-dimethyl-3-phenyl-4-[4-[2-pyridin-1-yl]ethoxy]phenyl]chromane hydrogencumarate) (Fig. 1) was synthesized at Amersham (Amersham, UK) and purified in the Department of Isotope Chemistry, Novo Nordisk A/S. The radiochemical purity was >99%, as determined by HPLC analysis, and the specific activity was 53 mCi/mmol. Nonradiolabeled levormeloxifene and chromatographic reference compounds were synthesised by Dr. S. Treppendahl and Dr. S. Madsen (Department of Chemistry, Novo Nordisk A/S, Maaloev, Denmark). Reference compounds included (+, −)-3, 4-trans-2, 2-dimethyl-3-phenyl-4-[(4-[2-pyridin-1-yl]ethoxy)phenyl]7-hydroxychromane hydrochloride; (+, −)-3, 4-trans-3-phenyl-4-[2-(pyrrolidin-1-yl)ethoxy]phenyl]-7-hydroxychromane hydrochloride; (+, −)-3, 4-trans-2, 2-dimethyl-3-phenyl-4-[4-(4-hydroxyphenyl)]-7-hydroxychromane hydrochloride; (+, −)-3, 4-trans-2, 2-dimethyl-3-phenyl-4-[4-(4-hydroxyphenyl)]-7-methoxychromane, with respective codes NNC 46–0002, NNC 46–0003, NNC 46–0004, and NNC 46–0005.

Animals and Dosing. Sprague-Dawley rats weighing 200 to 230 g were obtained from Charles River UK Ltd. (Margate, UK) or from Moehlbergs Breeding Laboratories (Lille Skensved, Denmark). Rats were housed in groups of six or fewer in stainless steel cages or singly in glass metabolism cages (Jencons Ltd., Leighton Buzzard, UK) in air conditioned rooms maintained at 19–23°C, 40 to 60% relative humidity, and a 12-h light/dark cycle. Rats were acclimatized under these conditions for at least 2 days before dosing.

[14C]Levormeloxifene was prepared by dissolution in 0.1 M H2SO4, [10% (v/v)] Tween 80, and injection grade water, and the pH of the solution was adjusted to 4 with sodium hydroxide. For excretion balance studies, three male and three female Sprague-Dawley rats received single oral dosages of [14C]-levormeloxifene (0.7 mg/kg or 50 mg/kg b.wt.), with each animal being administered approximately 25 μCi of radioactivity. The dose levels were selected based on previous pharmacokinetic and toxicokinetic studies. After dose administration, animals were housed in metabolism cages to allow the collection of urine and feces separately. At the end of 168 h the rats were sacrificed by cervical dislocation and the carcasses retained after removal of a selected number of organs/tissues.

For a disposition study, 15 male and 15 female Sprague-Dawley rats received a single oral dose of [14C]-levormeloxifene (0.7 mg/kg or 50 mg/kg b.wt.), with each animal being administered approximately 25 μCi of radioactivity, and at 2, 4, 24, 48, and 72 h, three male and three female rats had blood collected into heparinized tubes, plasma was prepared by centrifugation, and animals were sacrificed by cervical dislocation. A selected number of tissues were removed for additional analysis, including adrenal glands, brain, eye, heart, kidney, lacrimal gland, large intestine contents, large intestine wall, liver, lung, ovary (female), pancreas, pituitary, prostate (male), salivary gland, small intestine contents, small intestine wall, spleen, stomach contents, stomach wall, testis (male), thymus, thyroid, urinary bladder, and uterus (female). The final group of animals were used in the excretion balance experiment described above and were sacrificed at 168 h after dose administration. For whole body autoradiography (WBA) experiments, five male, five female, and five pregnant Sprague-Dawley rats received a single oral dose of [14C]-levormeloxifene (1.4 mg/kg b.wt., approximately 50 μCi per animal). At 2, 4, 24, 48, and 72 h animals were sacrificed and prepared for WBA.

For biliary excretion experiments, four male and four female Sprague-Dawley rats were surgically cannulated, resulting in animals with permanent bile fistulas as described by Remie et al. (1991) and Kuipers et al. (1985a,b). After recovery from surgery, collection of bile for up to 24 h post dose administration was performed. Dose levels and formulation were as described for excretion-balance experiments.

For pharmacokinetic experiments, levormeloxifene was dissolved in ethanol [1% (v/v)] and diluted with purified water. Final concentrations of dose material were 0.5 and 1 mg/ml for p.o. dosing. Female rats (n = 12 for each of two groups) received levormeloxifene at a dose level of 0.5 or 1.0 mg/kg b.wt., three times weekly for a period of 5 weeks, corresponding to the minimum dose levels where bone efficacy was observed.

Blood Sampling and Drug Analysis for Pharmacokinetic Experiments. Blood samples were collected before, 4, 6, 24, and 48 h after the sixth administration (day 13), and before, 4, 6, 24, 48, 54, 72, and 96 h after the final administration (day 34). In general, three blood samples, one on days 13 to 15 and two on days 34 to 38 (1000 μl each) were collected per animal by removal of blood from the ophthalmic venous plexus into heparinized Eppendorf tubes. Blood samples were centrifuged and the supernatant (plasma) aspirated and stored frozen (−18°C). Plasma (400 μl) was applied to mixed-mode columns (SPEC CS/SCX, 30 mg, 3 ml (Ansys, Irvine, CA) conditioned with 0.5 ml of methanol followed by 0.5 ml of phosphate buffer (0.1 M, pH 2.0). The cartridges were then rinsed with 0.5 ml of 1 M acetic acid, 0.5 ml of acetonitrile, and 0.5 ml of buffer in that order. The eluates were finally eluted from the cartridges by 1 ml of methanol-triethylamine (98:2, v/v). The eluate was evaporated to dryness in a TurboVap LV evaporator and redisolved in 100 μl of acetonitrile/water (40:60), and 75 μl was applied to the HPLC system.

The chromatographic system consisted of a Waters LC Module I System (Waters, Milford, MA), a Micro-Lab Universal-Thermostat Column Heater (Micro-Lab, Hoejbjerg, Denmark) and a Jasco 821-FP fluorescence detector (Jasco, Tokyo, Japan) operated at 279 and 305 nm excitation and emission wavelengths, respectively. The chromatograph was interfaced to an Expert Ease V 3.1 data system (Waters, Milford, MA) installed on a Digital Equipment Vax computer. The stationary phase consisted of a LichroSpher 100 RP-C18, 5 μm endcapped, 250 × 4 mm analytical column and a LichroSpher 100 RP-18, 5 μm endcapped, 4 × 4 mm precolumn, both supplied by Merck, Darmstadt, Germany. The mobile phase consisted of acetonitrile-ammonium formate (0.1 M, pH 3.3). The initial composition of the mobile phase was 40:60 of acetonitrile/ammonium formate. Within 10 min, the concentration of the organic solvent (acetonitrile) increased to 60% (linear increase). This concentration was maintained for 5 min, after which the concentration of acetonitrile declined to the initial value of 40% (linear decline in 5 min). For the purpose of equilibration this concentration (40%) was kept constant for 5 min. The flow rate was 1 ml/min, column temperature was 60°C, and run time was approximately 25 min. The assay was validated with respect to linearity, precision, and accuracy (intra- and total assay), lower and upper limit of quantitation, recovery, and interference from endogenous substances. For both levormeloxifene and its major metabolite, 7-desmethyllevormeloxifene, the assay was found to be linear in the range 2.5 to 500 ng/ml. The lower limit of quantitation was set to 2.5 ng/ml (both analytes) as the intra-assay precision was 7.3 and 9.1% (c.v.) and intra-assay accuracy was 83 and 108% for levormeloxifene and 7-desmethyllevormeloxifene, respectively. The total (intra- + interassay) precision at 2.5 ng/ml was below 7.3% (c.v.), and the total accuracy ranging between 92 and 99% for the parent compound and its major metabolite. At 500 ng/ml (upper limit of quantitation) the c.v. was below 4.8% and the accuracy was between 98 and 99% for levormeloxifene and 7-desmethyllevormeloxifene, respectively. The recovery of levormeloxifene and 7-desmethyllevormeloxifene from plasma was 86 and 78%, respectively. No interference from endogenous substances were present at the retention time of levormeloxifene and 7-desmethyllevormeloxifene.

Data Handling for Pharmacokinetic Experiments. Mean concentration/time data were calculated and analyzed by noncompartmental methods using the software Topfit (version 2.0; Heinzel et al., 1993). The apparent maximal concentration (Cmax) and the corresponding time (tmax) were determined visually from the concentration-time profile. Calculation of terminal half-life (t1/2) was based on data obtained during days 34 to 38. The terminal half-life was calculated by means of log-linear regression using at least six data points. The total area under the plasma concentration versus time curve (AUC) was determined by the linear trapezoidal rule from time zero to last sampling point equal to or above the lower limit of quantitation, AUC, added as the residual area as estimated by log-linear extrapolation to infinity.
Sections were prepared using a 9400 Cryostat Microtome (Bright Instruments) for dose administration, essentially as described by Ullberg and Karrson (1981). Ifene (1.4 mg/kg b.wt.) and WBA was performed at 2, 4, 24, 48, and 72 h after DEF5 film (Kodak Ltd., Hemel Hempstead, UK) and freeze-dried in a Lyolab B freeze-drier (Life Sciences, UK). Albans, UK) and the spinal cord. Sections were mounted on Cellux tape (Aston Clinton, St. Albans, UK) and the carcass, between the levels of the kidneys (males) or ovaries (females) and freeze-dried in a Lyolab B freeze-drier (Life Sciences, UK). The maximal concentration of radioactivity (microgram equivalents per gram) in selected tissues of the rat after administration of [14C]levormeloxifene (mean ± S.D.) for a dose of 0.7 mg/kg and 50 mg/kg is shown in Table 1.

### Table 1: Maximal Concentration of Radioactivity

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Male (0.7 mg/kg)</th>
<th>Female (0.7 mg/kg)</th>
<th>Male (50 mg/kg)</th>
<th>Female (50 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach wall</td>
<td>1.275 ± 0.349</td>
<td>1.132 ± 0.342</td>
<td>112.1 ± 44.8</td>
<td>370.9 ± 371.2</td>
</tr>
<tr>
<td>Small intestine wall</td>
<td>3.917 ± 1.455</td>
<td>4.428 ± 0.232</td>
<td>210.1 ± 43.4</td>
<td>236.9 ± 24.7</td>
</tr>
<tr>
<td>Large intestine wall</td>
<td>0.594 ± 0.169</td>
<td>0.843 ± 0.152</td>
<td>81.34 ± 11.37</td>
<td>68.95 ± 22.22</td>
</tr>
<tr>
<td>Liver</td>
<td>2.741 ± 0.158</td>
<td>4.004 ± 0.342</td>
<td>115.7 ± 5.5</td>
<td>189.6 ± 5.01</td>
</tr>
<tr>
<td>Lung</td>
<td>0.724 ± 0.039</td>
<td>0.928 ± 0.058</td>
<td>46.97 ± 2.57 g</td>
<td>64.53 ± 6.36</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.748 ± 0.397</td>
<td>3.503 ± 0.215</td>
<td>163.2 ± 20.8</td>
<td>207.1 ± 44.52</td>
</tr>
<tr>
<td>Plasma</td>
<td>0.051 ± 0.001</td>
<td>0.047 ± 0.015</td>
<td>2.57 ± 0.15</td>
<td>2.27 ± 0.24</td>
</tr>
</tbody>
</table>

*numbers in parenthesis refer to time (h) of maximal radioactivity.*

**Radioactivity.** Radioactivity in liquid samples (urine, plasma, bile, metabolism cage washes, and expired air trap solutions) was quantified by mixing aliquots with scintillation system MI-31 or Packard 200CATri-Carb (Packard Instruments Ltd., Pangbourne, UK). The products of combustion were absorbed in Optisorb I (Fisons plc, Loughborough, UK) and mixed with Optisorb S scintillator (Fisons plc). Large tissues were homogenized, with the addition of a known weight of water, using an Ultra-Turrax laboratory homogenizer [Semat Technical (UK) Ltd., St. Albans, UK]. Other tissues were finely scissor-minced or in the case of bone, ground with a pestle and mortar.

Radioactivity was measured by liquid scintillation counting using a Wallac 1409 or 1410 (Pharmacia-Wallac Oy, Turku, Finland) or a Packard 200CATri-Carb (Packard Instruments, Downers Grove, IL) automatic liquid scintillation analyzer with appropriate quench correction.

**WBA.** Sprague-Dawley rats received single oral doses of [14C]levormeloxifene (1.4 mg/kg b.wt.) and WBA was performed at 2, 4, 24, 48, and 72 h after dose administration, essentially as described by Ullberg and Larsson (1981). Sections were prepared using a 9400 Cryostat Microtome (Bright Instruments Co., Huntingdon, UK). Sagittal sections (30 µm) were cut at six levels through the carcass, between the levels of the kidneys (males) or ovaries (females) and the spinal cord. Sections were mounted on Cellux tape (Aston Clinton, St. Albans, UK) and freeze-dried in a Lyolab B freeze-drier (Life Sciences Laboratories Ltd., Luton, UK) before placing them in contact with Kodak DE85 film (Kodak Ltd., Hemel Hempstead, UK) and Brmax X-ray film (Amersham International, Amersham, UK). The film was exposed for 41 days at −20°C before its development. Autoradiographs were evaluated by visual inspection.

**Analysis of Metabolites.** Tentative identification of metabolites in fecal extracts, urine, plasma, and selected tissue extracts for the low and high doses was achieved by HPLC cochromatography with authentic standards. The elution times for levormeloxifene, NNC 46–0002, NNC 46–0003, NNC 46–0004, and NNC 46–0005 were typically 36, 22, 29, 32, and 55 min, respectively, although it was apparent on some of the HPLC runs that there was a shift in retention time, possibly due to the matrix in the injected samples. Additional identification was achieved by mass spectroscopy. Certain bile, urine, and plasma samples were deconjugated by mixing in equal proportions with β-glucuronidase (Type H1, 2000 U/ml) provided by Sigma, and incubating overnight at 37°C in acetate buffer (pH 5). Feces and tissue extracts were made by homogenization of samples in diethylether, centrifugation to obtain supernatants, evaporation of solvent, and resuspension in the HPLC mobile phase. Plasma samples (native or enzyme-treated) were applied to Isolute-HCX mixed-mode solid-phase extraction columns (3 ml/300 mg size; Microm科技, Amersham, UK) consisting of a pump, interface, and a UV 2000 variable wave.

**HPLC conditions.** Samples were analyzed using a LiChroplar 100 C18 column (particle size 5 µm, 250 × 4 mm id; Merck, Darmstadt, Germany) with a μBondapak C18 Guard-Pak precolumn (Millipore, Waters, MA). A Thermos Separation Products HPLC system was used (Thermo Separation Products, Stone, UK) consisting of a pump, interface, and a UV 2000 variable wavelength UV detector and a Ramona-5 or β-RAM on-line radioactivity detector.
Female rat: single oral dose

4 hours sacrifice

Fig. 4. WBA of a female rat 4 h after oral administration of \([14C]\)-levormeloxifene.

Quantitative Tissue Distribution. Radiolabeled levormeloxifene was distributed throughout body tissues after oral administration at dose levels of 0.7 and 50 mg/kg b.wt., and the rate of absorption and the general distribution of radioactivity was similar at both dose levels. The concentrations of radioactivity in the tissues increased in a dose-proportional manner. Not surprisingly, the greatest amounts of radioactivity were found in the gastrointestinal tract contents and in those organs responsible for absorption and elimination soon after administration (Table 1).

However, high concentrations of radioactivity were also found in the lungs. Maximal radioactivity concentrations were generally found 4 h postdose, but concentrations peaked in the testes and large intestine wall (50-mg/kg dose) of male rats at 24 h and in the fat (s.c.), lacrimal gland, large intestine wall, mammary gland, skin, and thymus of female rats at 24 h postdose. The proportion of the dose distributed into the tissues of female rats was greater than in male rats at all time points, at both dose levels (Fig. 2).

Excluding radioactivity in the gastrointestinal tract contents, radioactivity in the tissues of male rats reached a maximum level of 29.2 and 21.4% of the administered dose at 4 h and thereafter declined to 2.9 and 1.6% at 72 h postdose for the 0.7 mg/kg and 50 mg/kg b.wt. dose, respectively. Figure 3 shows the proportion of the dose in selected tissues, including the gastrointestinal tract contents, described in Materials and Methods. A higher proportion of radioactivity was retained in the tissues of female rats with a maximum level of 38.0 and 30.8% at 4 h, declining to 10.5 and 6.5% at 72 h postdose for the 0.7 mg/kg and 50 mg/kg b.wt. dose, respectively.

Peak radioactivity concentrations were generally 1.2 to 1.7 times greater in female rat tissues than in the corresponding male tissues with an even greater difference (2.2-fold) for fat observed. Mean peak concentrations in the whole blood, plasma, skin, small intestine wall, and stomach wall were similar between sexes. The concentration difference of radiolabeled levormeloxifene between male and female rats generally increased with time, and at 72 h the concentrations were 4- to 6-fold greater in the corresponding tissues of females than males. Even higher differences of 8-, 9-, and 23-fold were found for the testes and large intestine wall (50-mg/kg dose) of male rats at 24 h and in the fat (s.c.), lacrimal gland, large intestine wall, mammary gland, skin, and thymus of female rats at 24 h postdose. The proportion of the dose distributed into the tissues of female rats was greater than in male rats at all time points, at both dose levels (Fig. 2).

Mass spectrometry. Selected metabolites were analyzed by atmospheric pressure chemical ionization (APCI) mass spectrometry in both positive and negative ionization modes, where appropriate, using a TSQ7000 (Finnigan MAT, San Jose, CA) or an API 300 triple quadrupole liquid chromatography-tandem mass spectrometry (LC-MS-MS) mass spectrometer (Perkin-Elmer Sciex Instruments, Thornhill, Canada). Ionization conditions were optimized by varying the octopole offset (range –2.9 to –3.3 V in the positive ion mode, +3.0 V in the negative ion mode), capillary (+20 to +918 V in the positive ion mode, –20 to –968.8 V in the negative ion mode), and tube lens voltages (+72 to +181.5 V in positive ion mode, –72 to –187.1 V in negative ion mode). A portion of the sample (ca. 50 μl) was injected into the APCI interface at 1 ml/min, in acetonitrile/water (1:1, v/v). Mass spectra of the compounds of interest were recorded over an appropriate mass range for 2 min at a scan rate of 1 s/scan. An APCI mass spectrum of the compound of interest was obtained by averaging several scans across the region of the mass chromatogram where a response was observed, with appropriate background subtraction. This mass spectrum was examined to identify a candidate molecular ion, \([M+H]^+\) or \([M-H]^–\), for the compound of interest, and any other structurally significant fragment ions resulting from in-source collisionally induced dissociation. For metabolite identification experiments from bile, the mass spectrometer scanned in the range m/z 100 to 700 with a dwell time of 0.5 ms and a step size of 0.1 amu. The electrospray and orifice voltages were set to 5000 and 30 V, respectively. In the tandem mass spectrometry mode (product ion scan) the mass spectrometer scanned in the range m/z 50 to 500 (or higher, dependent on the mass of the precursor ion) with a dwell time of 0.5 ms and a step size of 0.1 amu. The electrospray and orifice voltages were set to 5000 and 30 V, respectively, and the fragmentation energy was +35 V.

Results

Quantitative Tissue Distribution. Radiolabeled levormeloxifene was distributed throughout body tissues after oral administration at dose levels of 0.7 and 50 mg/kg b.wt., and the rate of absorption and the general distribution of radioactivity was similar at both dose levels. The concentrations of radioactivity in the tissues increased in a dose-proportional manner. Not surprisingly, the greatest amounts of radioactivity were found in the gastrointestinal tract contents and in those organs responsible for absorption and elimination soon after administration (Table 1).
were not sampled in the quantitative analysis were the pineal body, brown fat, and the preputial, meibomain, and Harderian glands.

At 2 h after administration of $[^{14}C]$levormeloxifene (1.4 mg/kg b.wt.), the greatest radioactivity concentrations were found in the contents of the upper gastrointestinal tract, liver, lung, spleen, pancreas, adrenal gland, pineal body, and in the renal cortex. Slightly lower concentrations were found in the lower intestinal tract wall, lacrimal glands, salivary glands, thyroid, pituitary, and brown fat. Moderately high concentrations were found in the bone marrow, preputial gland, Harderian gland, myocardium, and prostate. Maximal concentrations in most tissues were noted at 4 h postdose, in agreement with the quantitative results. Concentrations of radioactivity in tissues of female rats were markedly higher than concentrations in corresponding tissues of male rats, indicating a slower rate of elimination in the female rat. This was substantiated in a separate toxicokinetic experiment (not shown) where the minimum trough concentrations of levormeloxifene were significantly higher in female rats than in males, at all sampling periods, after daily dosing for 28 days.

After 72 h radioactivity in the gastrointestinal tract was mainly localized to the lower part of the tract. Notably high concentrations were still observed in the Harderian gland and in the meibomian gland.

Photographic enlargements of the brain of female rats showed that radioactivity was distributed relatively uniformly throughout the cerebrum, cerebellum, and olfactory lobe (Fig. 5). Higher concentrations were observed in the pineal body and in the pituitary gland, and in the choroid plexus shortly after administration.

$^{14}$C-Excretion. After oral administration of radiolabeled levormeloxifene (0.7- or 50-mg/kg dose), radioactivity was excreted pre-
dominantly in the feces (Table 2). Mean fecal excretion accounted for 68.5 to 64.1% of the administered dose during the first 48 h, and 96.8 to 96.1% of the dose at the end of 168 h for the low (0.7 mg/kg)- and high (50 mg/kg)-dose groups, respectively, with male and female data combined at each dose level. Approximately 1.0% of the administered dose during the first 48 h, and 96.8 to 68.5% of the administered dose during the first 48 h, and 96.8 to 68.5% of the administered dose during the first 48 h, and 96.8 to 68.5% of the administered dose during the first 48 h, and 96.8 to 68.5% of the administered dose during the first 48 h, and 96.8 to 68.5% of the administered dose during the first 48 h, and 96.8 to 68.5% of the administered dose.

Interestingly, a lower proportion of the dose was excreted in the urine (Table 2). Mean urinary excretion was more rapidly by males than by females (Table 2). In a comparison of male and female rats, at both dose levels radioactivity was excreted more rapidly by males than by females (Table 2). Interestingly, a lower proportion of the dose was excreted in the urine of males than females, 0.6 to 0.8% compared with 1.3 to 1.3% (low and high doses, respectively) over 168 h, respectively, and the amount retained by the carcasses was also lower in males at both dose levels.

After oral administration of [14C]levormeloxifene to bile-cannulated rats, 16 to 20% of the radioactivity was excreted into the bile from female rats within 24 h, at the low and high doses, respectively (Table 3). In male animals, 33 to 29% of the dose was excreted into bile during the first 24 h (low and high doses, respectively). Less than 5.0% (mean, male and female animals, both dose levels) and 0.5% (mean, male and female animals, both dose levels) of the total radioactivity was found in, respectively, feces or urine (Table 3). The majority of the radioactivity was retained in the body up to 24 h, with over 60% of the recovered radioactivity being detected in the animal carcasses.

Pharmacokinetics. After oral administration, Cmax was generally observed 6 h after dosing. Pharmacokinetic parameter estimates are presented in Table 4. The half-life of elimination was long (24 h) and a doubling in dose resulted in an approximate doubling in exposure.

Metabolite Profiling and Identification. A number of different radioactive metabolites were tentatively identified by HPLC analysis, with subsequent structural confirmation by LC-MS-MS.

Bile. Proportions of radioactive components were generally similar at both the low- and high-dose groups in male and female animals (0–24 h post dose). One major metabolite (M1), in addition to two minor metabolites (M2 and M4) were isolated from bile. Typical HPLC chromatograms of bile from male (a) and female (b) rats, 0 to 6 h after oral administration of 0.7 mg/kg b.wt. [14C]levormeloxifene, are shown in Fig. 6. Mass fragmentation patterns for four metabolites are shown in Table 5 and the proportions of identified metabolites in the bile samples analyzed are shown in Table 6. The remaining radioactivity was excreted into feces during the experimental time period. Bile isolated from both male and female rats (both dose levels) consisted mainly of 7-desmethyllevormeloxifene glucuronide (M1) at all time points, with the additional quantitatively minor metabolites being identified as the glucuronides of hydroxylevormeloxifene (M2) and levormeloxifene (M4). Glucuronidase treatment of bile resulted in the identification of the aglucans for the isolated metabolites (M3 shown for reference purposes).

The highest concentration of M1 was detected during the first 6 h post dose administration in males and accounted for 57.4 to 56.2% of the sample radioactivity (low- and high-dosing groups, respectively), whereas in females higher concentrations of M1 were seen during 6- to 12-h post dose administration (62.5 to 61.3% of the sample radioactivity in the low- and high-dose groups, respectively). Minor sex differences were observed for the proportion of hydroxylevormeloxifene glucuronide (M2).

Feces. Proportions of radioactive components and metabolite profiles were generally similar at both the low- and high-dose groups in male and female animals. At least 11 metabolites were detected in feces, based on fraction collection data and on-line radioactivity monitoring; however some of these metabolites remained unidentified during the study (Fig. 7, representative on-line HPLC chromatograms of feces from male (a) and female (b) rats, 0 to 24 h after oral
administration of 0.7 mg/kg b.wt. [14C]levormeloxifene). There was some shift in retention time during HPLC analysis but this was attributed to a matrix effect and was compensated for by chromatography with the authentic reference standards. Subsequent HPLC runs (minus reference standards) were then used for isolation of metabolites for LC-MS-MS analysis. Feces consisted mainly of 7-desmethyllevormeloxifene (M6, norlevormeloxifene) at all time points, but unchanged drug was the second most prevalent component (Fig. 7 and Table 6). Mass fragmentation patterns for six of the isolated metabolites are shown in Table 5. The proportions of identified metabolites (M5–M9, and levormeloxifene) in feces are shown in Table 6.

Unchanged drug in feces accounted for 7.3 to 9.9% of the total dose in male rats from 0 to 24 h, decreasing to 0.5 to 1.8% in the 24- to 48-h sample in the low- and high-dose groups, respectively. In female rats, unchanged drug accounted for 7.8 to 3.9% of the total dose in the 0- to 24-h fecal radioactivity but only 1.7 to 1.1% of the 24- to 48-h samples, in the low- and high-dose groups, respectively. Total fecal excretion of unchanged drug from 0 to 48 h in male rats and from 0 to 72 h in female rats amounted to 7.8 to 11.7% (low- and high-dose group, respectively) and 10.4 to 6.1% (low- and high-dose group, respectively) of the dose, respectively.

The major metabolite in feces, 7-desmethyllevormeloxifene (M6), accounted for 32.1 to 27.6% of the total dose in male rats in the low- and high-dose groups, respectively, from 0 to 48 h. In female rats, 7-desmethyllevormeloxifene isolated in feces accounted for 28.3 to 25.6% of the administered dose (0–72 h) in the low- and high-dose groups, respectively. A number of less quantitatively significant metabolites (M5, M7-M9) were also isolated and identified (Tables 5 and 6; Fig. 7).

**Urine.** After both 0.7 and 50 mg/kg b.wt. doses of [14C]-levormeloxifene, radioactivity excreted in urine, accounting for approximately 1% of the administered dose, was largely associated with chromatographically polar metabolites. There were marginal sex differences in the polar metabolites, although there were no differences between dosing groups. For example, polar components made up 91% of the total urinary radioactivity excreted from 0 to 24 h (Fig. 8A) in male rats after dosing at 0.7 mg/kg b.wt. In urine excreted by female rats 24- to 48-h postdose (0.7 mg/kg b.wt. dose), a slightly lower proportion of urinary radioactivity was associated with these polar components (about 73–75%) (Fig. 8B).

After incubation with β-glucuronidase, proportions of polar components decreased with a concomitant increase in the less polar components, most notably 7-desmethyllevormeloxifene (M6) and monohydroxylevormeloxifene (M5 and M7). Proportions of unchanged drug also increased after enzyme treatment from only 1.4 and 0.4% of sample radioactivity before treatment, in urine from males and females, respectively, to 19.7 and 8.3%, respectively, at the low dose level.

These data indicate that radioactivity excreted in urine is predominantly associated with glucuronic acid conjugates, including conjugates of 7-desmethyllevormeloxifene (the major fecal metabolite), parent drug, and monohydroxylevormeloxifene.

**Plasma.** Unchanged drug was the major radioactive component present in the systemic circulation, at both dose levels, 4 and 24 h after dose administration. At 4 h, unchanged drug in the low- and high-dose groups, respectively, accounted for 71.9 to 37.7 and 61.5 to 49.4% of plasma extract radioactivity in male and female rats, respectively. Figure 9 shows HPLC profiles of plasma from male (a) and female (b) rats 4 h after oral administration of 0.7 mg/kg b.wt. [14C]levormeloxifene. There was again some shift in retention time during analysis of the different samples; however, this was compensated for by inclusion of reference standards. Other metabolites present in the systemic circulation included monohydroxylevormeloxifene (M5), and the mi-

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**TABLE 5**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Metabolite Source</th>
<th>m/z</th>
<th>Product Ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>bile</td>
<td>620</td>
<td>444, 294, 151, 98</td>
</tr>
<tr>
<td>M2</td>
<td>bile</td>
<td>650</td>
<td>474, 269, 181, 98</td>
</tr>
<tr>
<td>M3</td>
<td>bile, treated*</td>
<td>458</td>
<td>267, 176, 143, 119, 98, 91, 71</td>
</tr>
<tr>
<td>M4</td>
<td>bile</td>
<td>634</td>
<td>458, 326, 252, 151, 112</td>
</tr>
<tr>
<td>M5</td>
<td>feces extract</td>
<td>474</td>
<td>283, 271, 192, 181, 143, 119, 98, 71</td>
</tr>
<tr>
<td>M6</td>
<td>feces extract</td>
<td>444</td>
<td>241, 192, 119, 98, 91, 71</td>
</tr>
<tr>
<td>M7</td>
<td>feces extract</td>
<td>474</td>
<td>283, 255, 215, 165, 135, 107, 98, 71</td>
</tr>
<tr>
<td>M8</td>
<td>feces extract</td>
<td>490</td>
<td>472, 326, 267, 229, 223, 165, 130, 112, 91, 87, 84, 69, 44, 239, 211, 159, 118, 98, 91, 71</td>
</tr>
<tr>
<td>M9</td>
<td>feces extract</td>
<td>430</td>
<td>267, 189, 175, 165, 119, 98, 91, 71</td>
</tr>
</tbody>
</table>

*sample treated with β-glucuronidase.
nor metabolites (not shown in Fig. 9), 7-desmethyllevormeloxifene (M6), desmethylnorlevormeloxifene (M9), and 7-desmethyllevormeloxifene glucuronide (M1, which increased over time). Additionally, an unknown metabolite was also evident at both levels at all time points. After 24 h the proportion of radioactivity associated with unchanged drug decreased, and proportions of metabolites correspondingly increased. Interestingly, greater concentrations of the parent drug remained in the circulation of female rats at increasing time reflecting a possible lower rate of metabolism.

**Discussion**

Paramount to the development of a suitable selective estrogen receptor modulator for the treatment of osteoporosis is an understanding of the distribution, metabolism, and excretion of the compound after oral administration to preclinical species, because the pharmacological profile of compounds that bind to estrogen receptors may be altered due to the formation of metabolites with higher estrogenic activity than the parent compound (Dodge et al., 1997).

The results from the current study indicate that within 2 h after oral dose administration, radioactivity was higher in all tissues than in the blood, indicating rapid distribution of levormeloxifene and/or metab-
olites. However, based on HPLC analysis of tissue extracts (liver, kidney, and lung), much of this radioactivity, up to 94% of the sample radioactivity in some tissue samples, was associated with parent compound (Fig. 10), as indeed was the radioactivity associated with the systemic circulation (Fig. 9). Peak radioactivity concentrations were generally achieved 4 h postdose in both male and female animals at dose levels of 0.7 and 50 mg/kg b.wt. Interestingly, peak radioactivity concentrations were generally circa 1.2 to 1.7 times greater in female rat tissue than in corresponding tissues from male animals (not shown), and this was also reflected in a slower elimination of radioactivity from female animals. The reason for these differences are unclear, although this may be related primarily to fundamental differences in the metabolism between sexes (Mugford and Kedderis, 1998). Alternatively differences in elimination rates may be related to the compound class, that of a selective estrogen receptor modulator and differences in tissue binding and discrete receptor interactions (Dodge et al., 1997) with respect to the distribution and concentration of estrogen receptors (Kuiper et al., 1997). However, after single oral doses any differences between the rate of elimination of drug is probably more likely attributable to sex differences in metabolism.

Radioactivity was slowly excreted into feces, presumably after conjugation of metabolites and excretion into bile (Tables 2, 3, and 6) with only approximately 1% of the administered dose being excreted via the renal route. The major metabolite isolated from feces was characterized by HPLC and LC-MS-MS as 7-desmethyllevormeloxifene (norlevormeloxifene), indicating a typical cytochrome P450 demethylation reaction on the methoxy group of levormeloxifene, and accounted for about 34 to 43% of fecal extract radioactivity and for about 25 to 33% of the dose. Unchanged drug was also excreted, mainly from 0 to 24 h, and accounted for about 6 to 12% of the dose. Together these two components accounted for approximately 50% of the radioactivity excreted in those feces samples analyzed. Additional metabolites isolated and identified by LC-MS-MS, and accounting for up to 5% of the excreted radioactivity in rat feces during the first 24 h included two separate monohydroxylevormeloxifene (hydroxylated on different benzene rings), and desmethylnorlevormeloxifene. The formation of this metabolite is highly unusual because the proposed structure would arise from C-demethylation and O-demethylation of levormeloxifene. C-demethylation is an unexpected metabolic reaction and in the absence of definitive evidence, the proposed structure should be regarded with caution. Additionally, a pyrrolidinone ring-opened metabolite of levormeloxifene was also isolated and identified. A proposed route of metabolism is shown in Fig. 11.

The pharmacokinetics of levormeloxifene were also determined in female rats, and drug measurements were performed on animals dosed with 1.0 and 0.5 mg/kg b.wt. levormeloxifene, reflecting the anticipated therapeutic dose range. Results indicated that $C_{\text{max}}$ was generally observed 6 h after dosing, and the AUC values increased fairly proportionally to the dose. The half-life of elimination was long, being approximately 1 day. The plasma levels of the major metabolite 7-desmethyllevormeloxifene were in all pharmacokinetic samples below the lower limit of quantitation, confirming tissue distribution experiments, indicating that parent compound was the major circulating species and the major species in tissues even though the major metabolite excreted was 7-desmethyllevormeloxifene.
In conclusion, it would appear that levormeloxifene is an orally active compound and it can be predicted that the main site of metabolism is in the liver, with the major excretion pathway of parent compound and metabolites being via the fecal route in rodents. There appeared to be some minor gender differences in the distribution, metabolism, and excretion of radioactivity. However, there were no apparent changes in metabolism between dose levels. Similar studies in monkeys and human volunteers have indicated a similar excretion pathway and the formation of a number of comparable metabolites to those found in the rat species (Mountfield et al., 1999). What has not been established during these preclinical studies is the effect of the long half-life of this compound (219 h in volunteers; B.K., personal communication) on the overall consequences of repeated administration to patients. Interestingly, a similar compound being developed for osteoporosis, idoxifene, also has a long half-life, and clinical development was recently stopped due to adverse events in the clinic (SCRIP, 1999, 2431, p21).

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References
Dodge JA, Lugar CW, Cho S, Short LL, Sato M, Yang NN, Spangle LA, Martin MJ, Phillips DL,


