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DISPOSITION, METABOLISM, AND EXCRETION OF THE ANTICANCER AGENT CRISNATOL IN THE RAT

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ABSTRACT:

Disposition and metabolism of crisnatol (^{14}C-labeled), a novel antitumor agent, was examined after po and iv administration to rats. After both routes of drug administration, there was rapid elimination of the administered radioactivity in the urine (6–12% of the dose) and feces (81–92% of the dose). The drug appeared to be rapidly absorbed after oral dose and there was substantial "first-pass" metabolism. Analysis of the excreta indicated extensive metabolism of crisnatol by the rat, with the intact compound being the major radiolabeled component in the feces (17–20% of dose). Intact drug was not present in urine. Biotransformation of crisnatol by the rat mainly involves oxidation and conjugation pathways. Hydroxylation and dihydrodiol formation in the chrysene ring and oxidation of the propanediol side chain resulted in the formation of the three major fecal metabolites. The principal metabolite in the urine was also a dihydrodiol. Concentrations of intact drug in each tissue assayed exceeded those in plasma, and in the lungs the tissue/plasma ratio approached 300 and 82 at 2 hr after iv and po doses, respectively.

Crisnatol (fig. 1), 2-[(6-chrysenylmethyl)amino]-2-methyl-1,3-propanediol (BW A770U mesylate), is a novel DNA intercalator of the arylmethylaminopropanediol class of antitumor agents (1). This lipophilic compound has displayed potent antitumor activity against an array of murine tumors. Against P388 murine leukemia, it consistently produced substantial increases in life span with frequent long-term survivors.[1] In addition, crisnatol was active against murine P388 sublines resistant to alkylating agents, antimetabolites, vinca alkaloids, and cisplatin, but not all sublines resistant to DNA intercalators (2). In the human tumor stem, cell assay activity was also seen against lung, colon, breast, melanoma, renal, and ovarian cancers (3).[1] The drug is currently undergoing phase-II clinical trials against selected tumors including lung, glioblastoma, melanoma, myeloma, and ovarian cancer. As an essential part of preclinical drug development, the absorption, tissue distribution, metabolism, and excretion of ^{14}C-labeled crisnatol was investigated in the rat, a species that has been employed in pharmacology and toxicology studies. In this report, we describe the results of these studies, including isolation and characterization of the major extractable urinary and fecal metabolites of crisnatol in the rat.

Materials and Methods

Materials. [^{14}C]Crisnatol mesylate, 2-[(6-chrysen(5,11-^{14}C)yl methyl] amino)-2-methyl-1,3 propanediol methanesulfonate, was synthesized by Dr. John Hill at Wellcome Research Laboratories (Chemical Development Labs.). The specific activity was 48.50 mCi/mmol, and the radiochemical purity was >98% as determined by two-dimensional TLC/autoradiography using silica gel 60 F-254 plates. BSTFA[2] was obtained from Pierce Chemical Co. (Rockford, IL) and DMSO-d_6 from Merck Sharp & Dohme Isotopes (St. Louis, MO). All solvents were HPLC grade (EM Science, Gibbstown, NJ). Precoated glass-backed analytical plates of silica gel 60 F-254 (0.25 mm) and silica gel 60 (230–400 mesh) were obtained from E. Merck (Darmstadt, FRG). Preparative silica gel plates, Uniplate-T (20 × 20 cm), were obtained from Analtech (Newark, DE) and reversed-phase plates, LKC18F (0.2 mm) from Whatman (Maidstone, UK). Aquasol-2 was obtained from DuPont-New England Nuclear Research Products (Boston, MA). Bond-Elut C_{18} cartridges (3 ml) were obtained from Analytichem International (Harbor City, CA). β-Glucuronidase, Glucurase (bovine liver), and sulfatase (type H-2, from *Helix pomatia*) were obtained from Sigma Chemical Co. (St. Louis, MO).

Animals and Dosing. Male Sprague-Dawley (Charles River, Raleigh, NC) rats (240 to 290 g) were used. The rats were housed individually in cages (nonbalance study animals), or in glass metabolism cages (Crown Glass Co., Inc, Somerville, NJ), which permitted the complete separation and collection of urine and feces (balance study animals). Animals were

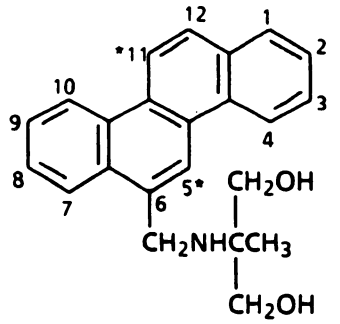

FIG. 1. *Chemical structure of crisnatol with * denoting the position of the ^{14}C-label.*

Portions of this work were presented at the Annual Meeting of The American Association of Cancer Research in 1986 and 1987.

[1] V. Knick, R. Tuttle, K. Bair, and D. Von Hoff: Arylmethylaminopropanediols (AMAPS); discovery, antitumor activity, and selection of clinical candidates. 5th NCI-EORTC meeting, October 22–24, 1986, The Netherlands.

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[2] Abbreviations used are: BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; EI/MS, Electron-impact/mass spectrometry; FAB, fast atom bombardment; TMS, trimethylsilyl; DMSO-d_6, dimethyl-d_6 sulphoxide.

fasted overnight (16 hr) prior to and for 4 hr after oral dosing, and urine and feces samples were collected daily over a 2-day period. Rats received a standard laboratory diet, with water allowed *ad libitum*, and were kept on a 12-hr light-dark cycle.

[^{14}C]Crisnatol and unlabeled compound were dissolved in 5% dextrose in water to give the desired specific activity for each experiment (2.5, 10, and 15 mCi/mmol for the oral dose balance, tissue distribution, and plasma profile studies, respectively). The drug was administered (5 mg/kg for each experiment) orally by gastric intubation or iv by tail vein injection. For serial sampling of blood after the oral dose, the jugular veins of three rats were cannulated the day before the study by a modification of the procedure described by Upton (4). Blood samples (0.3 ml) were obtained *via* the jugular cannula at 0.33, 0.67, 1, 2, 3, 4, 5, and 7 hr post-dose.

For the tissue distribution study, blood and tissue (liver, kidney, lung, brain, pancreas, skin) samples were collected from three rats each at 0.25, 0.5, 1, 2, 3, 6, 12, 24, and 48 hr after the iv dose. All tissues were rinsed in saline, and the tissues, plasma, urine, and feces were stored at −20°C until analysis. Urine and feces were collected from the three rats killed at 48 hr.

Radioactivity Measurements. All the samples were assayed for radioactivity with a Packard Tri-Carb model 460 CD liquid scintillation counter (Packard Instruments, Downers Grove, IL) and replicate determinations were made routinely. Portions (50–100 μl) of urine or plasma were mixed with 10 ml of Aquasol-2 scintillation fluid and counted directly for radioactivity. Tissue and feces samples (homogenized in water) were combusted using a Packard Tri-Carb model 306 sample oxidizer (Packard Instruments), and the radioactivity was determined by liquid scintillation counting. The oxidizer efficiency, which was routinely checked by combusting standards of known radioactivity, averaged 98% in all analyses; therefore, no correction factor was used.

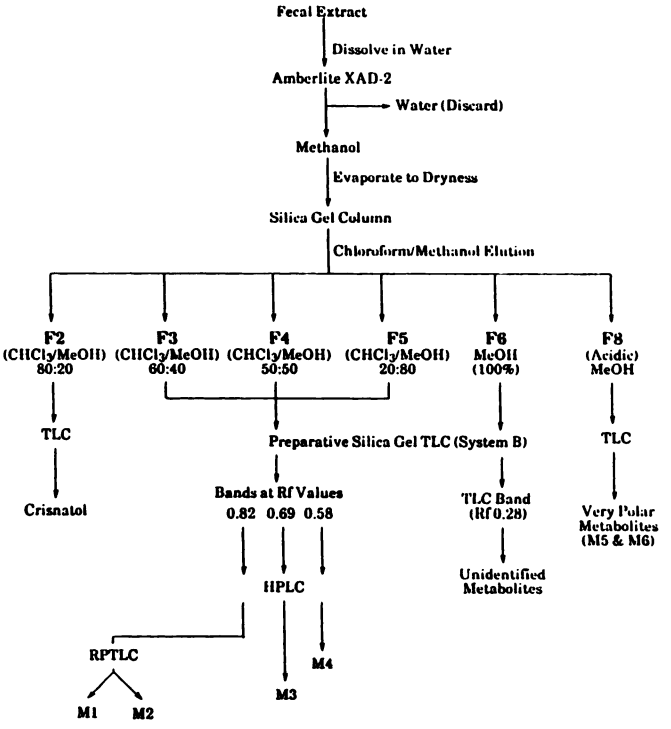
Analysis of Intact Drug and Metabolites. Crisnatol was extracted from plasma and tissue samples with 10% methanol in acetonitrile. Urine samples (0–24 hr) were concentrated by lyophilization. Fecal samples (0–24 hr) were extracted sequentially with methanol and a methanol/water mixture (1:1). The extracts were combined and reduced to dryness using a rotary evaporator.

Analytical TLC was performed on glass plates (20 × 20 cm) precoated with 0.25 mm silica gel 60. Two solvent systems were used to resolve crisnatol and its metabolites: plasma and tissue extracts were chromatographed in chloroform/methanol/water (25:10:1) (system A), and urine and fecal extracts were chromatographed in dichloromethane/methanol/ammonium hydroxide (14.8 M)/water (65:30:1:3) (system B). TLC plates were developed for 14 cm, and the radioactive bands were located on the plate by autoradiography using X-ray film (Kodak SB 5). The concentrations of crisnatol and metabolites were determined by scraping the corresponding ^{14}C-bands directly into scintillation vials, followed by scintillation counting of silica gel mixed with a small volume (0.4 ml) of water.

HPLC. The HPLC system consisted of an LDC pump (Constametric III) connected to a WISP 712, Waters autoinjector. The separation of metabolites was performed on a 5-μm Supelcosil LC-8-DB semi-preparative column (10 mm × 25 cm; Supelco, Inc., Bellefonte, PA), and the eluent was monitored at 269 nm using a Spectroflow 757 variable wavelength UV detector (Kratos, Ramsey, NJ). Crisnatol metabolites **M1, M2, M3,** and **M7** were purified using an acetonitrile/0.1% phosphoric acid (70:30) mobile phase pumped at a flow rate of 6 ml/min. Metabolite **M4** was chromatographed using an acetonitrile/1% formic acid/tetrahydrofuran (75:20:5) mobile phase pumped at 8 ml/min.

Isolation of Crisnatol Metabolites from Urine. Metabolites of crisnatol were isolated from the urine of rats dosed orally with [^{14}C]crisnatol (5 mg/kg). Pooled rat urine (0–24 hr) was passed through an Amberlite XAD-2 resin column that was washed with water and then a methanol/acetone (1:1) mixture. Approximately 86% of the radioactivity applied to the column was recovered in the organic wash and 4% in the aqueous wash, which was discarded. The volume of the organic fraction was reduced to dryness using a rotary evaporator, and the residue was redissolved in a small volume of methanol and chromatographed on preparative silica gel TLC plates (Uniplate-T; 20 × 20 cm) (Analtech, Newark, DE) in solvent system B. Autoradiograms of the developed plates revealed three major bands at R_F values of 0.67, 0.38, and 0.14. The silica gel corresponding to each band was removed and the radioactive compounds were eluted from the silica gel with methanol, which was evaporated using a rotary evaporator. The residues from bands at R_F values of 0.67 and 0.38 were dissolved in appropriate HPLC mobile phases (see HPLC section) and chromatographed. The band at 0.67 R_F gave a single radioactive peak. Repetitive injections were made and eluent fractions corresponding to this peak were combined and concentrated, to yield metabolite **M7**. The band at R_F of 0.14 was dissolved in water and applied to a 3-cc Bond-elut (C_{18}) solid-phase disposable column that had been preconditioned by washing with methanol followed by water. The column was eluted with water, methanol/water mixtures [20:80, 40:60, and 60:40 (v/v)], and methanol. Most of the radioactivity was eluted with methanol/water mixture (40:60), which was evaporated under a gentle stream of nitrogen at 40°C to yield metabolite **M8**. A portion of this purified metabolite was dissolved in 0.2 M acetate buffer, pH 5.0, and subjected to hydrolysis with β-glucuronidase (bovine liver, Glucurase). The hydrolysis product was analyzed subsequently by proton NMR.

Isolation of Crisnatol Metabolites from Feces. Metabolites of crisnatol were isolated from rat feces as shown in scheme 1. Pooled rat fecal samples (0–24 hr) were lyophilized and then sequentially extracted with methanol (3 times) and 10% methanol in water (once). This procedure removed approximately 65% of the ^{14}C present in the feces. The extracts were combined, and the methanol was evaporated using a rotary evaporator. The resulting aqueous phase was extracted with an equivalent volume of hexane, which was discarded, and chromatographed on an Amberlite XAD-2 resin column. The column was eluted with water followed by methanol. The majority (82%) of the ^{14}C applied to the column was recovered in the methanol fraction. The volume of the methanol fraction was reduced to approximately 5 ml using a rotary evaporator, and the fecal metabolite extract adsorbed onto Celite 545. Chloroform was added to this residue and the resulting slurry applied onto a silica gel 60 (230–400 mesh) column packed in chloroform. The column was first washed with chloroform (200 ml), followed by chloro-



SCHEME 1

ISOLATION OF CRISNATOL AND METABOLITES FROM FECES.

form/methanol (200 ml) mixtures [90:10, 80:20, 60:40, 50:50, 20:80 (v/v)], methanol, and finally 1% formic acid in methanol. Elution with 20% methanol in chloroform gave fraction **F2**. Similarly, 40%, 50%, and 80% methanol in chloroform and methanol (100%) gave metabolite fractions **F3, F4, F5,** and **F6,** respectively. The column was finally stripped with 1% formic acid in methanol, which gave fraction **F8.**

Fractions **F3, F4,** and **F5** were combined, and all of the above fractions were evaporated using a rotary evaporator at 40°C. The residues were redissolved in a small volume of methanol and chromatographed on preparative silica gel TLC plates (Uniplate-T; 20 × 20 cm) in solvent system B. Fraction **F2** gave one major TLC band (R_F = 0.89) which co-chromatographed with the intact drug. TLC/autoradiograms of the combined fractions **F3, F4,** and **F5** revealed three major bands at R_F values of 0.82, 0.69, and 0.58. Fraction **F6** had a major polar band at R_F of 0.28. Fraction **F8** had two very polar bands at R_F values of 0.11 and 0.15. The silica gel corresponding to each band was removed and ^{14}C was eluted from the silica gel with a methanol/chloroform (80:20) mixture, which was reduced to dryness using a rotary evaporator. The residues from TLC bands at R_F values 0.82, 0.69, and 0.58 were reconstituted in appropriate HPLC mobile phases (see HPLC section) and chromatographed. Bands at R_F values of 0.69 and 0.58 each gave a single radioactive peak. Repetitive injections were made and fractions corresponding to each peak were collected and concentrated to yield fecal metabolites **M3** and **M4,** respectively. During HPLC analysis of the band at R_F of 0.82, two radioactive peaks were observed by liquid scintillation counting of fractions collected at frequent intervals, indicating the presence of two drug-related components. However, the compounds were not well resolved. Thus, eluent fractions corresponding to these peaks were collected and reduced to dryness. The mixture was redissolved in a small volume of methanol and applied to a 0.2-mm reversed-phase TLC plate (LKF C_{18}), which was developed to 14 cm in methanol/water (80:20) solvent system. This procedure resolved the TLC band into two metabolites, **M1** (87%) and **M2** (13%), which were eluted from the reversed-phase matrix with methanol; the relative quantities of each were determined by liquid scintillation counting of the methanol fraction. These procedures afforded metabolite fractions (scheme 1) that were subsequently identified by GC/MS and proton NMR.

Spectral Data. EI/MS of the TMS derivatives of crisnatol and its metabolites were obtained using a VG 70-70S mass spectrometer (VG Analytical Ltd, Manchester, UK) after GC separation. The spectra were recorded at an ionization energy of 70 eV. The GC was performed using a 30-m DB-1 capillary column (J&W, Folsom, CA), which was temperature-programmed from 100°C to 300°C at 20°C/min. The column was held at 100°C for 2 min after injection. The FAB-mass spectrum of metabolite **M8** was obtained on a VG 70SQ mass spectrometer using glycerol as the matrix material. The TMS derivatives of crisnatol and metabolites were formed by heating the metabolite sample with approximately 0.5 ml of BSTFA at 100°C for 30 min. The reaction mixture was then taken to dryness under vacuum and reconstituted with BSTFA (5–10 ml). All mass spectra (both EI and FAB) were recorded on a VG 11-250J data system using VG-written acquisition software. Proton NMR spectra were recorded either on a GE GN-500 (500 MHz) or on a Varian XL-300 (300 MHz) spectrometer in DMSO-D_6 solution at ambient temperature.

Enzymatic Hydrolysis. To representative 0–24 hr rat urine and fecal extracts (dissolved in water), an equal volume of 0.2 M acetate buffer, pH 5.0, was added and each sample divided into three portions. The first portion was used as a control, the second portion was mixed with 100 μl of β-glucuronidase (bovine liver), and the third portion contained 500 μl of 0.4 M D-saccharic acid-1,4-lactone (inhibitor for β-glucuronidase) together with 15 μl of β-glucuronidase/arylsulfatase (type H-2 from *H. pomatia*). The samples were incubated at 37°C for approximately 16 hr and then applied to a 3-cc Bond-elut (C_{18}) disposable column, which was eluted sequentially with water and methanol. The methanol wash was examined by TLC/autoradiography in solvent system B.

Results

Excretion of Radioactivity in Urine and Feces. The excretion of radioactivity in urine and feces after single oral and iv doses of [^{14}C]crisnatol (5 mg/kg) to male rats is summarized in table 1. After oral administration, a mean of 96.9% of the administered radiocarbon was recovered from the excreta over a 48-hr period of collection, with the majority, 89.7%, recovered in the feces and 6.8% in the urine. Similarly, after iv administration, a mean of 83.2% of the administered radiocarbon was recovered in the feces and 10.7% in urine to give a total recovery of 93.9% over the same collection period. After both routes of administration, a total of about 84% of the radiocarbon was recovered during the first 24 hr.

Total Radioactivity and Crisnatol Profile in Plasma. After oral administration of [^{14}C]crisnatol, the plasma concentrations of total radioactivity reached a maximum at 2.0 hr and thereafter declined slowly (fig. 2*a*). Average peak crisnatol plasma concentrations of 0.1 μg/ml occurred approximately 1.0 hr after the oral dose and thereafter declined with a terminal half-life ($t_{1/2}$) of about 3 hr. After iv administration, the elimination of total radioactivity from plasma was at least biphasic, with a $t_{1/2}$ of about 13 hr (fig. 2*b*); the $t_{1/2}$ for crisnatol was 2 to 3 hr. After both routes of drug administration, about 50% of the total radioactivity in plasma at early times (0.25–0.33 hr) post-dose was accounted for by the intact drug. This proportion decreased with time, and by 6 to 7 hr intact drug was present in smaller amounts (7 to 16% of the plasma radioactivity).

Tissue Distribution. Radioactivity penetrated rapidly and extensively into tissues after iv administration (fig. 3*a*); 1 hr post-dose the concentration of total radioactivity in each of the tissues examined was higher than in plasma. The tissue/plasma concentration ratios of total radioactivity remained high for the 48-hr post-dose period. The concentration of intact drug was also higher in tissues compared to plasma (fig. 3*b*).

Crisnatol and Metabolite Pattern in Urine and Feces. TLC/autoradiographic analysis of urine and feces from male rats dosed orally with [^{14}C]crisnatol (5 mg/kg) showed several radioactive bands, indicating extensive metabolism of crisnatol (fig. 4). About 52% of the total radioactivity present in the urine (3% of the dose) was accounted for by two metabolites represented by bands at R_F values of 0.67 (**M7**) and 0.38, and crisnatol was present in trace amounts (<0.7% of the total radioactivity in urine) (fig. 4*A*). Intact drug was the major component in feces, accounting for nearly 23% of the total radioactivity present (17% of the dose) (fig. 4*B*). Two major bands containing **M1 + M2** and **M3** accounted for about 20% and 13% of the total radioactivity present in the fecal extracts, respectively. The other six

TABLE 1

Urinary and fecal excretion of radioactivity after a single oral or iv dose of [^{14}C]crisnatol to male rats

Values represent mean ± SD of three rats.

Route	Dose	Sample	% Administered Radioactivity		
			0–24 hr	24–48 hr	0–48 hr
	mg/kg				
Oral	5	Urine	6.09 ± 1.19	0.71 ± 0.36	6.80 ± 1.33
		Feces	77.96 ± 15.45	11.78 ± 13.19	89.73 ± 2.30
iv	5	Urine	10.04 ± 1.25	0.70 ± 0.14	10.74 ± 1.31
		Feces	74.08 ± 5.59	9.12 ± 5.29	83.20 ± 2.12

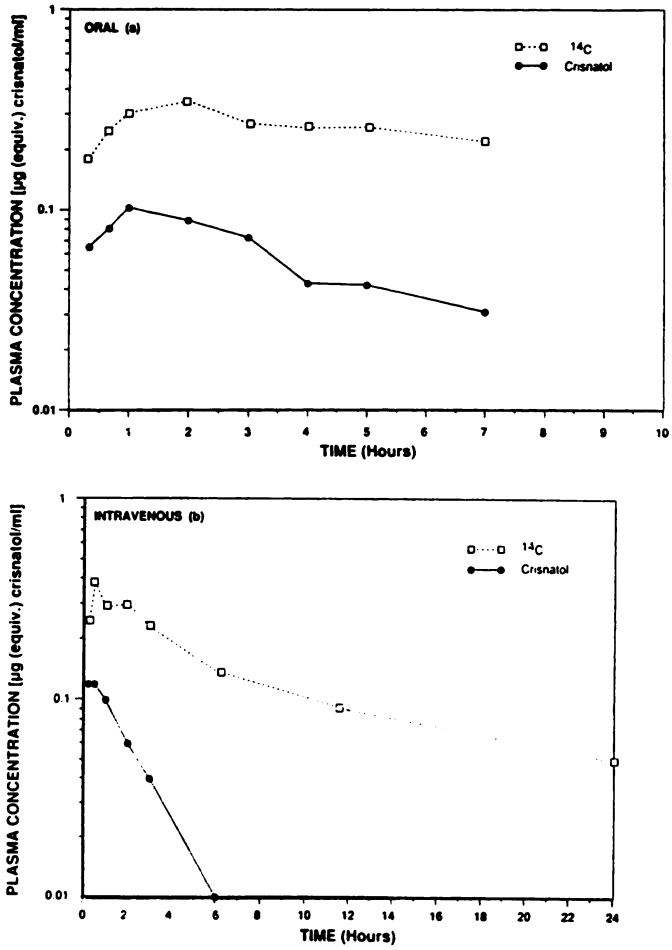


FIG. 2. *Plasma concentrations of total radioactivity and crisnatol in rats after a single oral (a) or iv (b) dose of [^{14}C]crisnatol (5 mg/kg).*

Values represent mean of three rats per time point.

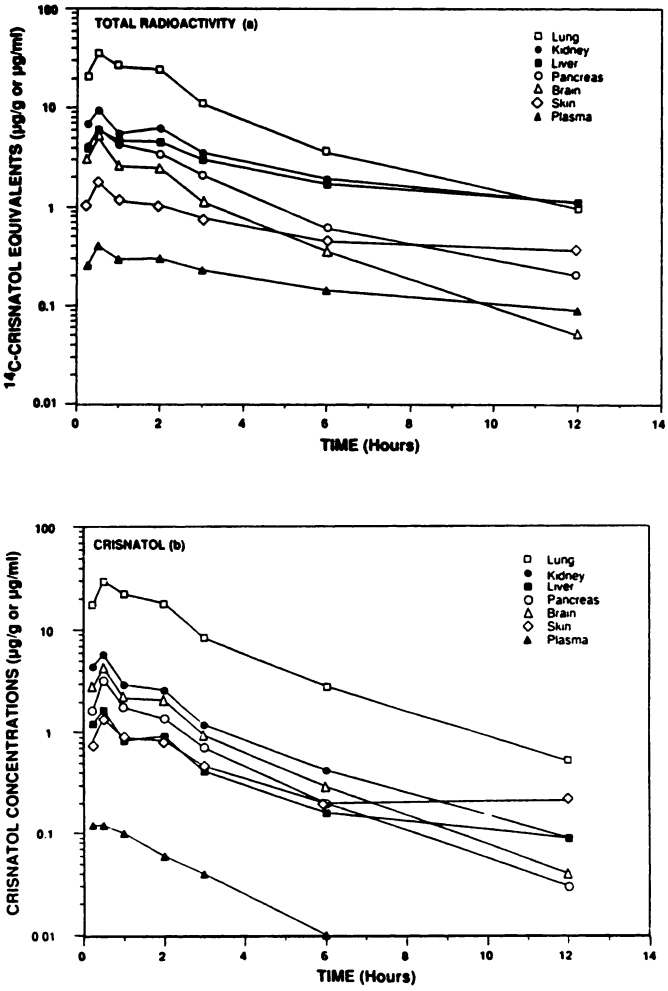


FIG. 3. *Concentrations of total radioactivity (a) and crisnatol (b) in rat tissues after a single iv administration of [^{14}C]crisnatol (5 mg/kg).*

Values represent mean of three rats per time point.

fecal metabolite species were present in smaller amounts, ranging from 3.5 to 7.1% of the total fecal radioactivity (fig. 4*B*). After treatment of the urine and fecal extracts with arylsulfatase, radioactivity associated with the TLC zone corresponding to **M6** decreased and the radioactivity corresponding to the crisnatol band increased, suggesting the presence of a sulfate conjugate of the parent drug. Similarly, treatment with β-glucuronidase suggested that the material **M8** in urine and **M5** in feces were both glucuronic acid conjugates of **M1**.

Identification of Metabolites. Metabolites isolated from urine and feces of rats dosed orally with [^{14}C]crisnatol were characterized by GC/MS and proton NMR. Selected EI/MS and proton NMR data of crisnatol and metabolites are summarized in tables 2 and 3, respectively.

Crisnatol Isolated from Feces. The EI/MS of the TMS derivatives of the synthetic standard and the crisnatol isolated from feces were identical. Two intense fragment ions at *m/z* 386 and 241 correspond to the loss of CH_2OTMS from the side chain and the loss of the side chain at the methylamino bond, respectively. The molecular ion was not observed; however, a characteristic ion, the loss of a methyl group was seen at *m/z* 474. The TLC, GC, and HPLC chromatographic properties of the isolated intact drug agreed well with those of the standard compound.

Metabolite M1. In fecal extracts, crisnatol metabolites **M1** and **M2** migrated as a single TLC band (fig. 4*B*) with **M1** being the major (87%) component. The EI/MS of **M1** (fig. 5*A*) gave a molecular ion at *m/z* 577, indicative of the addition of 88 mass units to the TMS derivative of crisnatol (*m/z* 489). This was consistent with the gain of an oxygen atom and a hydroxycrisnatol structure. The major ion at *m/z* 329 corresponds to the loss of the side chain at the methylamino bond. Additional characteristic ions at *m/z* 474 and 562 were due to the loss of CH_2OTMS and CH_3 radical groups, respectively, from the parent ion.

The position of the hydroxyl group was determined from the 500 MHz ^{1}H-NMR spectrum (fig. 5*B*). Analysis of chemical shifts and coupling patterns in the aromatic region (7.7–9.2 ppm) indicated that the hydroxylation occurred at either C-1 or C-2 on the chrysene ring. Strong evidence for substitution at C-1 came from the downfield shift of the 12-proton and upfield shift of the 11-proton (table 3). The highfield 2-proton at 7.0 ppm shows a clear *ortho* pattern, while the 3-proton at 7.5 ppm shows a di*ortho* pattern.

Metabolite M2. In the EI/MS of **M2**, ions higher than *m/z* 418 and its associated isotope peaks were not observed. High resolution EI/MS led to assignment of the formula $C_{25}H_{30}O_2Si_2$ to the molecular ion at *m/z* 418, and the fragmentation pattern (table 2) indicated that the structural modification occurred both on chrysene ring and the propanediol side chain. These data are

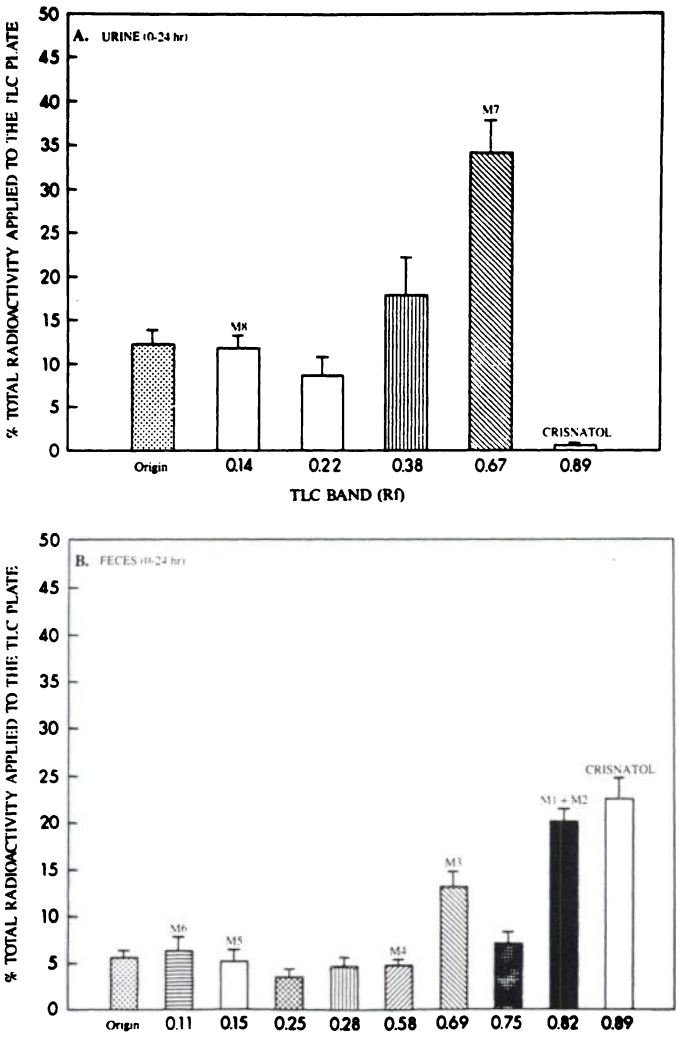


FIG. 4. *TLC profile of crisnatol and metabolites in urine (A) and feces (B) from rats dosed orally with [^{14}C]crisnatol (5 mg/kg).*

Each *bar* represents mean ± SD of four rats.

consistent with a monohydroxy crisnatol structure with a modification of the side chain to a hydroxymethyl group. These findings were confirmed by desorption chemical ionization/mass spectrometry. The aromatic region of ^{1}H-NMR spectrum for **M2** was similar to that of **M1**. The same downfield shift of 12-proton and upfield shift of 11-proton were observed (table 3). The high field proton 2, at 7.0 ppm, shows the same *ortho* doublet and the 3-proton at 7.5 ppm, the same di*ortho* pattern. The major difference in the spectrum was the upfield shift of the 7-proton, which indicated a possible change in the side chain at C-6. These data support the proposed structure shown in fig. 7.

Metabolites M3 and M7. **M3** and **M7** gave identical EI/MS (fig. 6). Similarly, the chromatographic properties (TLC, HPLC, GC) and ^{1}H-NMR spectra of fecal metabolite **M3** were identical with those of urinary metabolite **M7**. The observed molecular ion, *m/z* 667, indicated the addition of 178 mass to the TMS derivative of crisnatol (*m/z* 489), consistent with the addition of two oxygen, two hydrogen atoms, and two TMS groups. This suggested a dihydrodiol structure for **M3** and **M7**. The location of the hydroxyl groups was based on the correlation spectroscopy spectrum (data not shown) and the observed chemical shifts and coupling constants from the normal proton spectrum. The chemical shifts of 1- and 12-protons (table 3) indicated that the two hydroxyl groups were at either the C-1 and C-2 positions or the C-3 and C-4 positions. ^{1}H-NMR data on similar compounds (5, 6) showed that if the hydroxyl groups were at C-3 and C-4 of the "bay region" of the chrysene ring, the hydroxyl groups would be *trans*-diaxial due to crowding in the bay. This would mean that the 3- and 4-protons would be diequatorial, and their coupling constant, $J_{3,4}$, would be 3–5 Hz. If, however, the hydroxyl groups were at the 1- and 2-positions, a *trans*-diequatorial conformation of the hydroxyl groups would result. This would make 1- and 2-protons diaxial and result in a coupling constant with $J_{1,2}$ of 10–11 Hz. Examination of ^{1}H-NMR and correlation spectroscopy spectra for **M7** shows the coupling constant is 11 Hz, establishing that the two hydroxyl groups are located at C-1 and C-2. These findings indicate that **M3** and **M7** are both *trans*-1,2-dihydro-1,2-dihydroxycrisnatol (fig. 7).

Metabolite M4. Examination of the EI/MS of the TMS derivative of **M4** revealed the presence of a parent ion (M^+) at *m/z* 503 (loss of two hydrogen atoms, and addition of one oxygen atom) to TMS derivative of crisnatol (mol wt 489). This was consistent with the oxidation of the propanediol side chain from an alcohol (R – CH_2OH) to a carboxylic acid (R – COOH). Characteristic fragment ions at *m/z* 400 and 386, corresponding to the loss of CH_2OTMS and CO_2TMS from the molecular ion, respectively, were present (table 2). In addition, an intense ion at *m/z* 241, derived from the loss of the side chain at the methylamino bond, was observed. The ^{1}H-NMR spectrum of this metabolite showed a marked similarity to that of the parent drug, except for the 0.28 ppm upfield shift of the 5-proton. All other resonances showed only slight changes in chemical shift, with no missing peaks (table 3). The data indicate that the

TABLE 2

Selected EI mass spectral data of crisnatol and its metabolites (TMS derivatives)

Compound	*m/z* Value (Relative Intensity) and Proposed Ion Structure				
Crisnatol (standard)	474 (1.0) $[M - CH_3]^+$	386 (29.16) $[M - CH_2OTMS]^+$	241 (100.0) $[M - NHCH_3(CH_2OTMS)_2]^+$	73 (19.0) $Si(CH_3)_3^+$	
Crisnatol (feces)	474 (0.9) $[M - CH_3]^+$	386 (60.0) $[M - CH_2OTMS]^+$	241 (100.0) $[M - NHCH_3(CH_2OTMS)_2]^+$	73 (19.0) $Si(CH_3)_3^+$	
M1	577 (0.1) $M^{+\cdot}$	562 (0.9) $[M - CH_3]^+$	474 (30.5) $[M - CH_2OTMS]^+$	329 (100.0) $[M - NHCH_3(CH_2OTMS)_2]^+$	73 (95.7) $Si(CH_3)_3^+$
M2	418 (31.6) $M^{+\cdot}$	329 (14.5) $[M - OTMS]^+$	256 (7.5) $[M - OTMS - TMS]^{+\cdot}$	239 (7.2) $[M - OTMS - TMSOH]^{+\cdot}$	73 (100.0) $Si(CH_3)_3^+$
M3 and M7	652 (1.0) $[M - CH_3]^+$	564 (41.8) $[M - CH_2OTMS]^+$	419 (30.0) $[M - NHCH_3(CH_2OTMS)_2]^+$	329 (10.5) $[m/z\ 419 - TMSOH]^+$	73 (100.0) $Si(CH_3)_3^+$
M4	503 (0.2) $M^{+\cdot}$	488 (0.8) $[M - CH_3]^+$	400 (5.6) $[M - CH_2OTMS]^+$	386 (11.8) $[M - CO_2\ TMS]^+$	73 (27.0) $Si(CH_3)_3^+$
	256 (6.8) $[M - CCH_3(CH_2OTMS)(CO_2TMS)]^+$			241 (100.0) $[M - CCH_3(CH_2OTMS)(CO_2TMS)]^+$	

TABLE 3

Proton NMR data for crisnatol and its metabolites

Compound	Aromatic Protons and Chemical Shift (ppm) in DMSO-d_6										
	1	2	3	4	5	7	8	9	10	11	12
Crisnatol (standard)	8.16	7.78	7.78	8.95	9.11	8.43	7.83	7.83	9.08	8.95	8.20
Crisnatol (feces)	8.17	7.78	7.78	8.97	9.12	8.43	7.84	7.84	9.09	8.93	8.20
M1		7.05	7.53	8.38	8.80	8.32	7.70	7.70	8.91	8.75	8.31
M2		7.06	7.54	8.32	8.80	8.22	7.71	7.71	8.93	8.76	8.3
M3 and M7	4.70	4.36	6.24	7.36	8.41	8.29	7.75	7.75	8.92	8.79	7.94
M4	8.08	7.68	7.68	8.86	8.83	8.50	7.75	7.75	8.95	8.92	8.09
M8		7.39	7.77	8.59	9.08	8.41	7.84	7.84	9.09	8.93	8.59

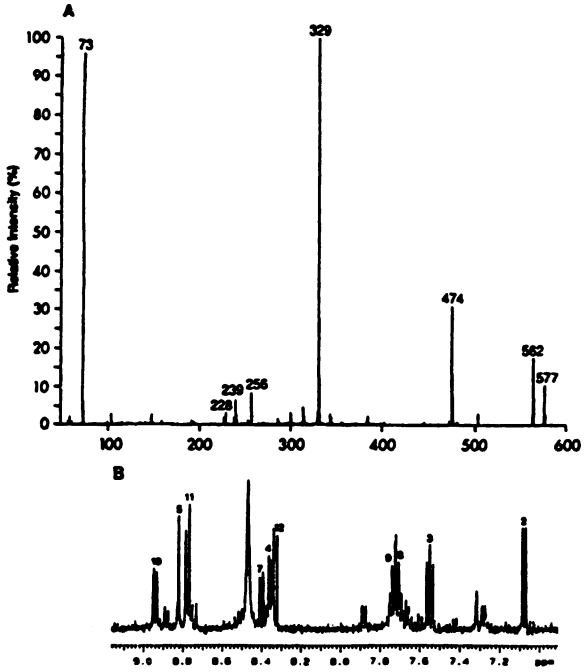


FIG. 5. *EI/MS (A) and ^{1}H-NMR (B) spectra of the major crisnatol metabolite M1 isolated from the feces.*

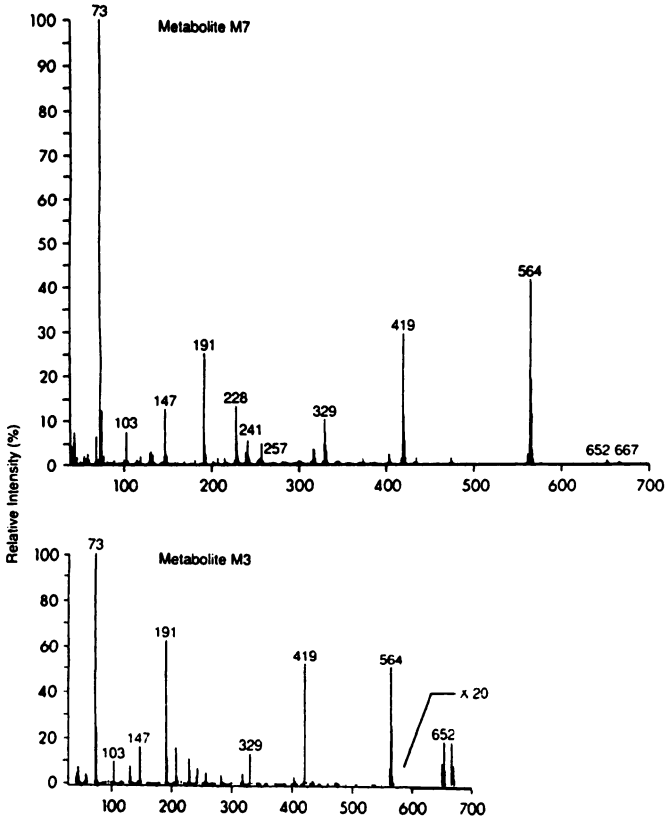


FIG. 6. *EI/MS spectra of TMS derivatives of crisnatol metabolites M3 and M7.*

propanediol side chain was modified, but the chrysene ring was intact. These findings support the structure shown in fig 7.

Metabolite M8. FAB/MS analysis, which does not require sample volatilization, gave a spectrum that was consistent with **M8** being a glucuronide conjugate of **M1**. A positive ion FAB/MS spectrum consisted of the $(M + H)^+$ ion at m/z 538, $(M + H + G)^+$ at m/z 630, and $(M - NHCCH_3(CH_2OH)_2)$ at m/z 433. Comparison of the ^{1}H-NMR spectra of **M8** (before and after treatment with β-glucuronidase) with that of metabolite **M1** (1-hydroxycrisnatol) indicated that the glucuronic acid conjugation occurred with the OH group at the C-1 position on the chrysene ring, yielding a phenolic glucuronide.

Discussion

After oral and iv administration of [^{14}C]crisnatol to male rats, the major route of elimination of the radioactivity was *via* the feces (83 to 90%). This suggests that the radioactivity present in the feces after an oral dose was largely due to biliary excretion of drug and/or metabolites, rather than to unabsorbed drug, since only 23% of the total radioactivity present in the feces was due to intact drug. Molecular size is an important determinant of biliary excretion of compounds, and in rat the existence of a molecular weight threshold (325 ± 50) is well documented (7). Therefore, it is not surprising that for crisnatol (mol wt 345), biliary (and ultimately fecal) excretion was the predominant pathway. Thus, oral doses of crisnatol are well absorbed by the male rats, but by comparison of the plasma profiles of the total radioactivity and parent drug after oral administration, there appears to be extensive "first-pass" metabolism (fig. 2*a*).

TLC/autoradiographic analysis of urine and fecal extracts revealed at least eight distinct radioactive bands (fig. 4), indicating that crisnatol was extensively metabolized by male rats. The proposed metabolic pathway of crisnatol in rats is summarized in fig. 7. Intact drug was the major fecal component, accounting for approximately 17 and 20% of the administered dose after oral and iv administration, respectively. The compound was primarily metabolized by oxidation and conjugation pathways. Hydroxylation at the C-1 position of the chrysene ring resulted in the formation of the major fecal metabolite, **M1**. Dihydrodiol formation in the chrysene ring (**M3**), hydroxylation of the chrysene ring and oxidation of the cleaved side chain (**M2**), and

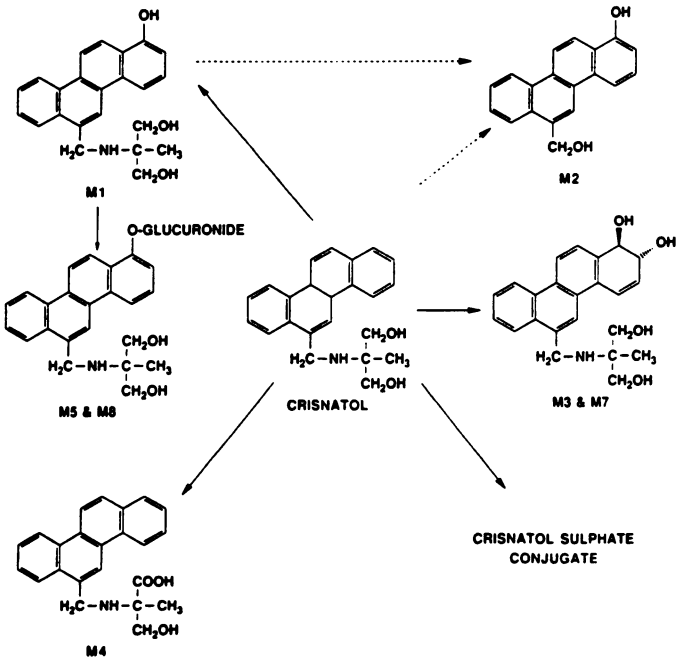


FIG. 7. *The proposed metabolic pathway of crisnatol in rats.*

oxidation of the propanediol side chain (M4) accounted for the major fecal metabolites. The primary urinary metabolite, M7 (2% of the dose) was also a crisnatol dihydrodiol derivative. Treatment of urine and fecal extracts with β-glucuronidase/arylsulfatase indicated the presence of a glucuronide conjugate of the hydroxylated metabolite (M1) and a sulfate conjugate of the parent drug.

In beagle dogs, like rats, the major route of elimination of crisnatol was *via* the feces (between 71 and 83% of the dose) after oral and iv doses (5 mg/kg); thus, biliary excretion is significant in that species also (8). Analysis (TLC/autoradiography) of the fecal excreta clearly showed that the drug was extensively metabolized by the dog and at least two major metabolic products present in the feces (representing between 47 and 58% of the total radioactivity) had the same TLC chromatographic properties as the 1,2-dihydrodiol (M3) and 1-hydroxy (M1) crisnatol metabolites formed by the rat (fig. 7). These two metabolites are also the major products formed when crisnatol is incubated with human liver microsomes.[3] These findings suggest that the rat, dog, and human appear to metabolize crisnatol *via* two major common pathways. Formation of isomeric dihydrodiol metabolites of chrysene, a compound structurally similar to crisnatol (substituted chrysene), has been reported with rat liver microsomes (9, 10), but in the present study there was no evidence of formation of significant quantities of crisnatol dihydrodiol isomers, except the 1,2-dihydrodiol.

After iv administration of [^{14}C]crisnatol (5 mg/kg) to rats, the radioactivity was rapidly and extensively taken up by all tissues examined; at 0.25 hr post-dose, the distribution of intact drug was lung ≫ kidney > brain ≥ pancreas ≥ liver ≥ skin (fig. 3*b*). A similar tissue distribution profile was observed also in the rat at 2 hr after the oral dose. This general rank order held through the 12-hr period after iv dose, except that intact drug in skin persisted at high concentrations relative to other tissues and, at 12-hr post-dose, only lung had higher concentrations of [^{14}C] crisnatol. During the first 6 hr post-dose, an average of 86% of the radioactivity in brain, 82% in lung, and 69% in skin was intact drug. The percentage of total radioactivity at early time points that was intact drug averaged 33% in liver and 63% in kidney; by 12-hr post-dose, these proportions decreased to 8% in both tissues.

Tissue/plasma concentration (T/P) ratios of intact drug reflected the extensive tissue distribution of this compound in rats. Averaged over the first 12-hr post-dose, the T/P ratios in kidney, brain, pancreas, and liver were 34, 22, 16, and 13, respectively. Skin averaged a T/P ratio of 11 for the first 6-hr post-dose, but at 12 hr this ratio had increased to 54, indicating binding and/or slow drug efflux from this site relative to plasma. The ability of the rat lung to concentrate basic lipohilic drugs is well established (11), and it appears to be dose- and species-dependent (12). The average lung-to-plasma concentration ratio for crisnatol (a lipophilic compound with a pka of about 8.1) was 300 and 82 at 2 hr after the iv and oral dose, respectively. Thus, lung cancer would appear to be one of the selective disease targets for crisnatol therapy in humans, a hypothesis that is currently being investigated.

[3] D. K. Patel, et al: Metabolism of a novel antitumor agent, crisnatol, by the human hepatoma cell line, HepG2, and hepatic microsomes: characterization of metabolites. Submitted for publication.

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