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Drug Metabolism

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TITLE: Identification of three new human blood metabolites of Ro 10-9359

SUMMARY: Metabolites were isolated from an extract of pooled blood from psoriatic patients on chronic therapy with Ro 10-9359 by HPLC and identified by mass spectrometry and nuclear magnetic resonance spectroscopy. The two major metabolites in the extract were the all-trans acid (Ro 10-1670) and the 13-cis acid (Ro 13-7652), which had been previously identified as human blood metabolites of Ro 10-9359. Three new blood metabolites were identified. One metabolite corresponded to Ro 13-7652 with a hydroxy group on the aromatic methyl on carbon 2 (Ro 23-4750). Two other metabolites showing strong 280 nm absorption but not 365 nm absorption had acid side chains that were shortened by one carbon and had a reduced 11,12 double bond. One of these two metabolites retained the aromatic methoxy group at carbon 3 (Ro 23-4293) while the other metabolite was the phenolic analog (Ro 23-3571). The latter compound had previously been identified in glucuronidase-treated bile of a human volunteer after a single dose of Ro 10-9359. The biological activities of these new metabolites are not known.

Complete for pre-clinical studies.

Study performed at: Hoffmann-La Roche Inc., Nutley, NJ 07110.

Identification of three new human blood metabolites of Ro 10-9359.

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Drug Metabolism

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INTRODUCTION

Ro 10-9359 (etretinate, Scheme 1) is a synthetic retinoid that is undergoing clinical evaluation as an oral agent for treatment of severe psoriasis (1, 2, 3, 4). The pharmacokinetics and metabolism of Ro 10-9359 have been reviewed (5, 6, 7).

Hänni et al. (8) found that about 15% of a 100 mg oral dose of 10,11-³H labelled Ro 10-9359 administered to two psoriatic patients was excreted in the urine during the first four days. Fecal excretion during this period contained 75% of the administered radioactivity with unchanged Ro 10-9359 accounting for 80% of the fecal radioactivity. Fourteen urinary metabolites, representing 73% of the urinary radioactivity, were identified. All of these metabolites had side chains shortened by 4, 6, or 9 carbons (8). Ro 10-1670, the free acid of Ro 10-9359, was identified as the major metabolite in the plasma (8).

In a recent study Ro 10-9359 labelled with ¹⁴C at carbon 7 was administered as a 100 mg oral suspension to six normal volunteers and two volunteers with biliary t-tubes. During the two weeks following administration, the six normal volunteers excreted a mean \pm SD of $31.1 \pm 9.4\%$ and $51.1 \pm 13.8\%$ of the administered radioactivity in the urine and feces, respectively (9). The percent

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of radioactive dose excreted by the two t-tube subjects during five days after dosing was 11.5 and 8.6% in the urine, 31.2 and 53.1% in the feces, and 8.4 and 7.3% in the bile (10). The two major metabolites in the β -glucuronidase-treated bile were identified by Bugge' et al. (11) as Ro 11-8492, a tetranor phenolic acid, and Ro 23-3571, a tetrahydro, mononor phenolic acid (Scheme 1). In addition, small amounts of Ro 10-1670 and Ro 12-7310 (the phenolic analog of Ro 10-1670) were detected in the β -glucuronidase-treated bile samples (11).

In addition to Ro 10-1670, another major metabolite in plasma of psoriatic patients after 2 months of chronic Ro 10-9359 therapy was identified by Hänni et al. (12) as Ro 13-7652, the 13-cis isomer of Ro 10-1670. In most psoriatic patients Ro 13-7652 is the major drug-related component in blood during chronic treatment with Ro 10-9359 (13). It is also the major drug-related component in human blood 24 hours after a single oral dose of Ro 10-9359 (9, 13, 14, 15).

During the HPLC analyses of blood extracts from psoriatic patients on chronic Ro 10-9359 therapy, we observed that the chromatograms (365 nm UV detection) frequently showed a peak corresponding to an unknown polar metabolite. This report describes the isolation and identification of this polar metabolite, as well as two other metabolites which were detected at 280 nm.

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EXPERIMENTAL

Materials

All reference retinoids were provided by Hoffmann-La Roche Inc. N-Methyl-N-nitroso-p-toluenesulfonamide (DiazaId) was obtained from Aldrich Chemical Co. Inc., Milwaukee, WI. Solvents were purchased from the following suppliers: methanol and acetonitrile ("distilled in glass"), Burdick and Jackson Laboratories, Inc., Muskegon, MI; ethyl ether (anhydrous AR), Mallinckrodt Chemical Works, St. Louis, MO; glacial acetic acid (Ultrex), J.T. Baker Chemical Co., Phillipsburg, NJ. Reagents were of analytical grade. Phosphate buffer (1M, pH 6.5) was prepared by titrating 1M KH_2PO_4 (68.1 g/500 ml) with 1M K_2HPO_4 (34.8 g/200 ml) until pH 6.5 was reached.

Laboratory Precautions

Because of the facile photodecomposition of retinoids, all handling of the samples was carried out in darkened rooms equipped with yellow lights, and amberized glassware was used whenever possible.

Blood Samples

Blood samples were obtained from psoriatic patients on chronic treatment with Ro 10-9359 (50-100 mg daily in divided doses). The study was conducted at the University of Washington, Seattle, WA, by Dr. T. Franz, Department of Dermatology (Protocol 2366A). Whole

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blood was collected in 10-ml evacuated containers (BD Vacutainers containing sodium heparin, Cat. 6527, Becton-Dickinson Co., Rutherford, NJ). The blood was transferred to polypropylene tubes fitted with screw caps (Cat. 541, Walter Sarstedt Inc., Princeton, NJ) for storage at -70°C . All samples had been previously analyzed for Ro 10-9359, Ro 10-1670, and Ro 13-7652 by a reversed-phase gradient-elution HPLC method (14) and the results will be reported elsewhere (13). Those samples which contained relatively large amounts of the unknown polar metabolite (365 nm) were pooled. The samples had been stored 8-14 months at -70°C .

Control blood from healthy volunteers was collected in heparinized containers and stored at -20°C .

Metabolite Isolation

Pooled patient blood (310 ml) was mixed with 200 ml of phosphate buffer (1M, pH 6.5) and extracted three times with 400 ml of ethyl ether in a 2-liter separatory funnel. A second identical 310-ml pool of blood was similarly extracted. The combined ether layers from the two extractions were evaporated just to dryness on a water bath at 37°C with a stream of nitrogen. The residue was transferred to a 50-ml centrifuge tube with 20 ml of acetonitrile. Ten ml of phosphate buffer (1M, pH 6.5) was added and the sample was extracted three times with 30 ml of ethyl ether. The sample was centrifuged

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to aid in the separation of the ether layer. The combined ether layers were evaporated to almost dryness as described above. The residue was mixed with 10 ml of acetonitrile which resulted in the formation of a white precipitate. The acetonitrile layer was removed and the remaining precipitate was washed twice with 10 ml of acetonitrile. The acetonitrile fractions were combined and concentrated to 1 ml.

The concentrated extract was chromatographed in two separate runs as described below. Fractions corresponding to the major peaks from the two runs were combined and evaporated to dryness with a stream of nitrogen. Each residue was treated with diazomethane to form methyl esters as described below. Each methylated sample was purified by HPLC and stored in acetonitrile at -17°C. Immediately before NMR analysis, the major portion of each solution was evaporated to dryness and the residue was dissolved in the NMR solvent. The smaller portion was evaporated to dryness and dissolved in 20 µl of methanol for mass spectral analysis.

A 30-ml sample of control blood was extracted and analyzed by HPLC in a manner similar to that described for the patient blood.

HPLC

The following conditions were used for the isolation of the metabolites and the purification of the isolated metabolites after treatment with diazomethane.

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The modular HPLC system consisting of a glass dual-chamber gradient-forming apparatus, a Rheodyne model 7120 injector, and a Micromeritics model 750 pump has been described previously (16). The mixing chamber contained 120 ml of methanol/0.01M ammonium acetate/acetic acid, 50:50:0.25 (v/v/v). The reservoir contained 145 ml of methanol/0.2M ammonium acetate/acetic acid, 95:5:0.04 (v/v/v). A concave gradient was generated and the flow rate was 4 ml/min. A guard column (7 cm x 1.5 mm ID) packed with Permaphase ODS (30 μ m particles) and a Zorbax ODS column (25 cm x 9.4 mm ID, 6 μ m particles) (both from Dupont Instruments, Wilmington, DE) were used at room temperature. The eluate was monitored at 280 and 365 nm with Laboratory Data Control Spectro Monitor II and III detectors, respectively; the detectors were connected to a Gould Brush 105 2-pen recorder. Eluate fractions corresponding to the peaks of interest were collected manually.

Derivatization

Methyl esters were prepared by treating dried samples with 1-2 ml of ethereal diazomethane which was prepared from N-methyl-N-nitroso-p-toluenesulfonamide according to the alcohol-free method given on the Diazald label. After 5 min at room temperature, the solutions were evaporated to dryness under a stream of nitrogen. Under these conditions, carboxyl groups were methylated, but phenols were not.

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Identification Techniques

Mass spectra were obtained on a Varian MAT CH5 or a VG ZAB-1F spectrometer at 70 eV. Samples were introduced with the direct insertion probe.

Proton nuclear magnetic resonance spectra (NMR) were obtained in deuteriochloroform with tetramethylsilane as the internal standard on a Varian XL-200 instrument in the Fourier-transform mode with a 45° flip angle and a 3 sec repetition rate.

RESULTS

In a preliminary experiment, the amount of the unknown polar metabolite observed in the 365 nm chromatogram of extracts of patient blood was not increased by the treatment of the blood with Glusulase. Therefore, untreated blood was used for metabolite isolation.

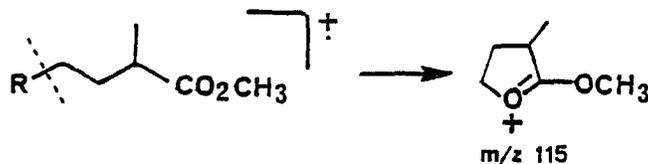
The 280 and 365 nm chromatograms of the extract of pooled blood from psoriatic patients receiving Ro 10-9359 are shown in Figure 1. A methanol/water mobile phase system was used to isolate the metabolites as initial studies with an acetonitrile/water mobile phase system resulted in coelution of peaks A and B. The large peaks at 52 and 62 min were also present in the chromatograms of the control blood extract (not shown), suggesting that they are endogenous blood constituents. The retention time and NMR spectral data of the peak at 62 min were compatible with retinol.

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Each of the peaks labelled A-F in Figure 1 was collected, combined with the corresponding peak from the injection of the second half of the blood extract, and treated with diazomethane to convert acid groups to methyl esters. These samples were purified by HPLC before spectral analyses. The retention times of peaks A, B, C, D and E increased by 9-13 minutes, indicating that these metabolites contained an acid group which was methylated by the diazomethane treatment. Other smaller peaks in Figure 1 that were possibly drug-related were not isolated.

Peak A. This peak did not absorb at 365 nm, suggesting that the conjugated side chain had been altered. The mass spectrum of the methyl ester of peak A showed a molecular ion at m/z 316 (Table 1). Fragment ions at m/z 149 and 187 indicated that peak A had a phenolic structure (Scheme 2, line a). The m/z 115 fragment ion (Table 1) suggested that the terminal portion of the side chain was saturated:

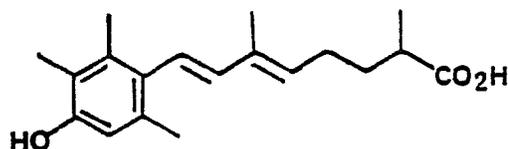


Tables 1 and 2 show that the mass and NMR spectra of the methyl ester of peak A were very similar to the spectra obtained previously

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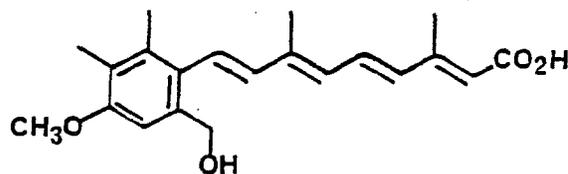
for a deconjugated human biliary metabolite of Ro 10-9359 that was identified as a tetrahydro, mononor phenolic acid (Ro 23-3571, Scheme 1) (11). In addition, peak A and the bile metabolite coeluted when extracts of blood and hydrolyzed bile were coinjected. Therefore peak A and the bile metabolite have the same structure.



Peak A
Ro 23-3571

Peak B. The strong absorption of peak B at 365 nm indicated that the side chain was intact. The NMR spectrum of the methyl ester of peak B (Table 2) showed a two-proton signal at δ 4.78 (-CH₂O-) and only two aromatic methyl groups, which suggested that one of the aromatic methyl groups had been hydroxylated. The chemical shifts of the methyl on C13 (δ 2.1) and the proton on C14 (δ 5.67) were compatible with a 13-cis side chain. The mass spectrum of the methyl ester of peak B (Figure 2) showed a molecular ion at m/z 356 and fragment ions at m/z 177 and 217 (Scheme 2, lines d and e) which were also compatible with an acid metabolite with a hydroxy group on the aromatic portion of the molecule.

The position of the hydroxy group in peak B was determined by comparison of its spectral data to that of reference Ro 16-2001,



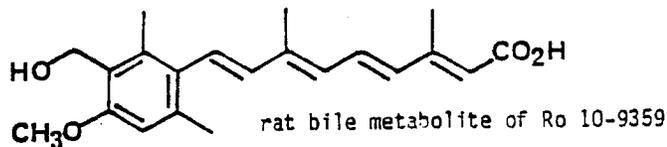
Ro 16-2001

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the 5-hydroxymethyl analog of Ro 10-1670. The mass spectrum of the methyl ester of Ro 16-2001 was similar to that of the methyl ester of peak B (Table 1) except for a very strong fragment ion at m/z 201 in the spectrum of Ro 16-2001-ME. Possibly the m/z 201 ion was formed by the loss of water from a $[d+H]^+$ type fragment ion (Scheme 2). The chemical shifts of the aromatic methyl groups in the NMR spectrum of Ro 16-2001-methyl ester (δ 2.19, 2.25; Table 2) are significantly different from the shifts of the aromatic methyl groups in the spectrum of peak B-methyl ester (δ 2.32, 2.36). Therefore, the hydroxy group of peak B is not on the 5-methyl. The methyl signal at δ 2.15-2.19 in the NMR spectra of the reference compounds (Table 2) is most likely due to the 2-methyl group (17). The absence of a similar signal in the spectrum of peak B-methyl ester suggests that the hydroxy group is on the 2-methyl.

Hänni *et al.* (8) proposed the following structure for a metabolite of Ro 10-9359 isolated from rat bile after enzymatic hydrolysis.

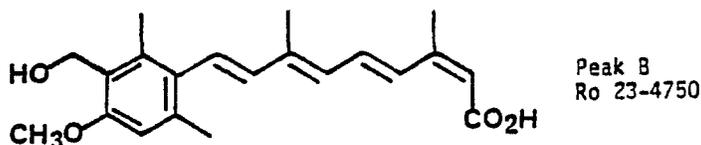


The NMR spectrum of the methyl ester of this rat bile metabolite (Table 2) is very similar to the NMR spectrum of the methyl ester

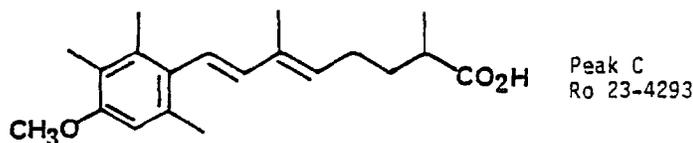
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of peak B except for the protons affected by the geometry of the 13,14 double bond, i.e., the protons on C12 and C14 and the methyl on C13. Therefore, the ring portions of the rat bile metabolite and peak B are most likely identical. Peak B is most likely the 13-cis analog of the rat bile metabolite as shown below.



Peak C. As indicated by its lack of absorption at 365 nm, peak C had an altered side chain. The mass spectrum of the methyl ester of peak C (Figure 2) showed a molecular ion at m/z 330. The fragmentation pattern was similar to that of the methyl ester of peak A (Table 1) except that most of the peak C ions were 14 daltons higher than the peak A ions. Except for the additional methyl group at δ 3.82 in the NMR spectrum of the methyl ester of peak C, it was very similar to that of the methyl ester of peak A (Table 2). Therefore, peak C is the methyl ether analog of peak A.



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Peaks D, E. Based on retention time (Figure 1), mass spectra (Table 1, Figure 2), and NMR spectra (Table 2), peaks D and E are Ro 13-7652 and Ro 10-1670, respectively (Scheme 1).

Peak F. The retention time (Figure 1), mass spectrum (Table 1), and NMR spectrum (Table 2) of peak F were compatible with unchanged Ro 10-9359.

Chromatograms (365 nm) from the analyses of blood samples from 14 psoriatic patients on chronic Ro 10-9359 dosing (some of whose blood was used in this study) were reexamined to determine the prevalence and amounts of Ro 23-4750 (peak B), the hydroxy acid metabolite. No significant amounts of this metabolite were observed in blood samples collected after a single 100 mg dose of Ro 10-9359, but it was present in nearly all blood samples collected during weeks 4-24 of chronic dosing. The concentration range of this metabolite was 20-100 ng/ml (roughly estimated from the Ro 13-7652 calibration curve), which is about 30% of the concentration of Ro 13-7652, the major drug-related component in most of these samples.

DISCUSSION

The major metabolites of Ro 10-9359 identified in human blood (8, 12), bile (11) and urine (8) are shown in Scheme 1. This study confirmed the presence of Ro 10-9359, Ro 10-1670, and Ro 13-7652

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in the blood of psoriatic patients. In addition three new metabolites were identified. The metabolite with strong UV absorption at 365 nm contained a 13-cis side chain and a hydroxyl group on the 2-methyl group. Most likely, this metabolite (peak B, Ro 23-4750) was formed by hydroxylation of Ro 13-7652. This metabolite is identical to a rat biliary metabolite identified by Hänni et al. (8) except that the rat metabolite had an all-trans side chain.

Two metabolites with no 365 nm absorption but strong 280 nm absorption were identified as mononor, tetrahydro acids. One contained the aromatic methoxy group (Ro 23-4293) while the other was the phenolic analog (Ro 23-3571). The latter compound had previously been identified in glucuronidase-treated bile of a human volunteer after a single 100 mg dose of Ro 10-9359 (11). The mechanism for the formation of these two metabolites is unknown, but presumably Ro 10-9359, after deesterification, undergoes oxidative decarboxylation to give a C₁₉ acid. The reduction of the 11,12 double bond of this C₁₉ acid to give Ro 23-4293 is an unusual metabolic pathway for mammalian enzymes. Possibly intestinal bacteria may be responsible for this reduction step (19). Metabolic demethylation of the aromatic methoxy group of Ro 23-4293 would give Ro 23-3571.

The contributions of the three new blood metabolites identified in this report to the biological activities of Ro 10-9359 are unknown. Several of the urinary metabolites of Ro 10-9359 with shortened

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side chains have been synthesized and were reported to be biologically inactive (5). Also, the reduction of the 7,8 or the 11,12 double bond of Ro 10-1670 resulted in decreased activity in a mouse skin papilloma test and reduced reversal of keratinized lesions in a tracheal organ culture system (18).

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Notebook

11025: 104-9, 116-120
11026: 190-193, 196-207, 235-236, 242, 244

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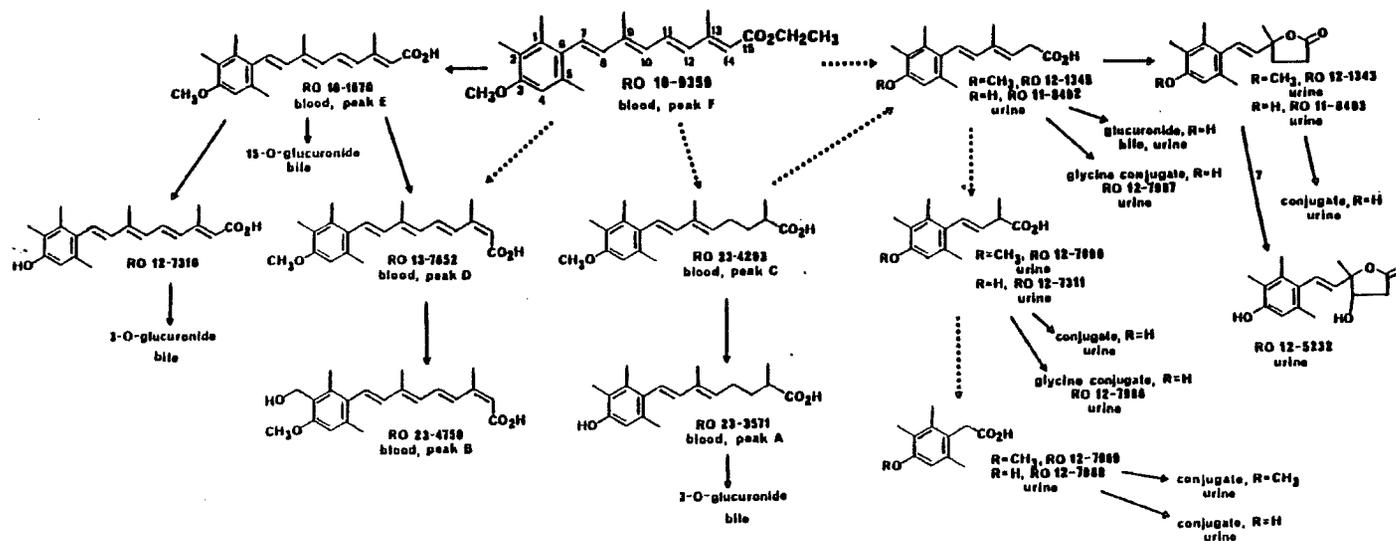
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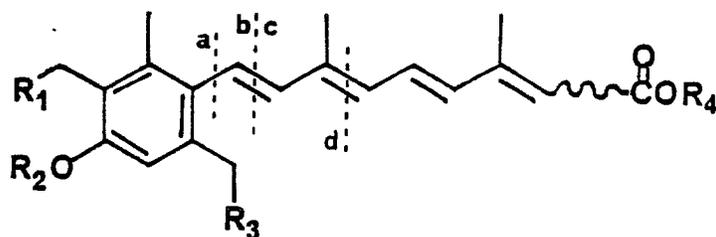
Scheme 1

Summary of the known human metabolites of Ro 10-9359.

The arrows indicate the possible pathways for formation of the metabolites, with the dotted arrows representing multiple metabolic steps.

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Substituents				Fragment Ions m/z					
<u>R₁</u>	<u>R₂</u>	<u>R₃</u>	<u>R₄</u>	<u>[a+H]⁺</u>	<u>[b+H]⁺</u>	<u>[c-H]⁺</u>	<u>[d-H]⁺</u>	<u>[d+H]⁺</u>	<u>[d+H-H₂O]⁺</u>
a) H	H	H	CH ₃	136	149	177	187	189	--
b) H	CH ₃	H	CH ₂ CH ₃	150	163	191	201	203	--
c) H	CH ₃	H	CH ₃	150	163	177	201	203	--
d) OH	CH ₃	H	CH ₃	--	--	177	217	--	--
e) H	CH ₃	OH	CH ₃	--	--	177	217	--	201

Scheme 2

Some postulated mass spectral fragmentations of Ro 10-9359, analogs, and metabolites.

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Table 1.

Mass Spectral Data of Ro 10-9359 Human Blood Metabolites
and Some Reference Compounds^a.

<u>Metabolite</u>	<u>M⁺</u>	<u>Major Fragment Ions</u>
A-ME ^b	316	269, 241, 215, 213, 199, 187, 149, 115
B-ME ^c	356	323, 297, 291, 279, 264, 263, 217, 177
C-ME ^c	330	283, 229, 227, 215, 213, 201, 199, 163, 150, 115
D-ME	340	325, 293, 281, 265, 251, 203, 201, 177, 163, 150
E-ME	340	325, 293, 281, 265, 251, 203, 201, 177, 163, 150
F	354	339, 293, 281, 265, 251, 203, 201, 163, 150
<u>Reference Compound</u>		
Ro 10-9359	354	339, 293, 281, 265, 251, 203, 201, 191, 163, 150
Ro 10-1670-ME	340	325, 293, 281, 265, 251, 203, 201, 177, 163, 150
Ro 13-7652-ME	340	325, 293, 281, 265, 251, 203, 201, 177, 163, 150
Ro 12-7310-ME	326	311, 279, 267, 251, 237, 189, 187, 177, 149, 136
Ro 16-2001-ME	356	338, 291, 279, 264, 263, 217, 201, 189, 177, 119, 105
<u>Other Metabolite</u>		
Human bile ^d (Ro 23-3571)	316	269, 241, 215, 213, 199, 187, 149, 115

a. The m/z values for the molecular ion (M⁺) and the major fragment ions with m/z >100 are given. The spectra of metabolites were obtained on the VG ZAB-1F, whereas the spectra of the reference compounds were obtained on the Varian MAT CH5.

b. ME = methyl ester.

c. The mass spectra of the methyl esters of peaks B and C are shown in Figure 2.

d. Data from Ref. 11.

Table 2.

Proton Magnetic Resonance Data of Ro 10-9359 (human Blood Metabolites and Some Reference Compounds).

Metabolites	Chemical Shifts (ppm)													Others
	Protons on:							Methyls on:						
	C4	C7	C6	C10	C11	C12	C14	C10	C20	C50	C9	C13	CO ₂ CH ₃	
A-ME	6.52 s	6.39 d	6.10 d	5.42 t	e	e	e	2.23 s	2.16 s	2.23 s	1.87 s	1.18 d	3.69 s	
B-ME	~6.7 s	~6.7 d	~6.3 d	~6.3 d	~7.0 dd	7.8 d	5.67 s	2.36 s	-- s	2.32 s	2.1 s	2.1 s	3.72 s	OCH ₃ :3.86s;OCH ₂ :4.78s
C-ME	6.60 s	6.42 d	6.11 d	5.42 m	e	e	-- s	2.23 s	2.15 s	2.28 s	1.88 s	1.19 d	3.69 s	OCH ₃ :3.82s
D-ME	6.62 s	6.69 d	6.27 d	6.32 d	7.02 dd	7.81 d	5.66 s	2.25 s	2.16 s	2.31 s	2.10 s	2.10 d	3.72 s	OCH ₃ :3.83s
E-ME	~6.6 s	~6.7 d	~6.3 d	~6.2 d	~7.0 dd	~6.3 d	5.78 s	2.24 s	2.15 s	2.30 s	2.11 s	2.38 s	3.72 s	OCH ₃ :3.82s
F	6.61 s	6.70 d	6.26 d	6.19 d	7.03 dd	6.32 d	5.79 s	2.24 s	2.15 s	2.30 s	2.11 s	2.38 s	-- s	OCH ₃ :3.83s;OCH ₂ :4.18q; CH ₃ :1.3t
<u>Reference Compound</u>														
Ro 10-9359	6.60 s	6.69 d	6.26 d	6.19 d	7.03 dd	6.32 d	5.78 s	2.23 s	2.15 s	2.30 s	2.11 s	2.37 s	-- s	OCH ₃ :3.83s;OCH ₂ :4.19q; CH ₃ :1.30t
Ro 10-1670-ME	6.61 s	6.70 d	6.25 d	6.20 d	7.04 dd	6.33 d	5.79 s	2.25 s	2.16 s	2.31 s	2.12 s	2.39 s	3.72 s	OCH ₃ :3.83
Ro 11-7652-ME	6.61 s	6.69 d	6.26 d	6.31 d	7.02 dd	7.81 d	5.66 s	2.24 s	2.16 s	2.30 s	2.10 s	2.10 d	3.72 s	OCH ₃ :3.83s
Ro 12-7310-ME	6.55 s	6.67 d	6.25 d	6.20 d	7.04 dd	6.33 d	5.80 s	2.25 s	2.18 s	2.25 s	2.11 s	2.38 s	3.73 s	OH:4.6s
Ro 16-2001-ME	6.89 s	6.75 d	6.34 d	6.23 d	7.02 dd	6.34 d	5.80 s	2.25 s	2.19 s	-- s	2.11 s	2.38 s	3.73 s	OCH ₃ :3.87s;OCH ₂ :4.69s
<u>Other Metabolites</u>														
Human bile ^c (Ro 23-3571)	6.52 s	6.39 d	6.10 d	5.42 t	1.8 m	e	2.50 m	2.22 s	2.16 s	2.22 s	1.88 s	1.19 d	3.69 s	
Rat bile ^d	6.64 s	6.65 d	6.24 d	6.20 d	7.03 dd	6.33 d	5.79 s	2.36 s	-- s	2.31 s	2.11 s	2.38 s	3.72 s	OCH ₃ :3.85;OCH ₂ :4.77d; OH:2.05t

^a Proton magnetic resonance data were obtained in deuteriochloroform. Structures are shown in Scheme 1 or Results section. Abbreviations are s = singlet, d = doublet, t = triplet, m = multiplet, dd = double of doublets, ME = methyl ester.

^b Tentative assignments of the chemical shifts of the aromatic methyl groups are based on the NMR spectra of Ro 10-9359 analogs (16).

^c data from Ref. 11.

^d Data from Ref. B; structure postulated as the C2-hydroxymethyl analog of Ro 10-1670.

^e Signal too weak or masked by other signals.

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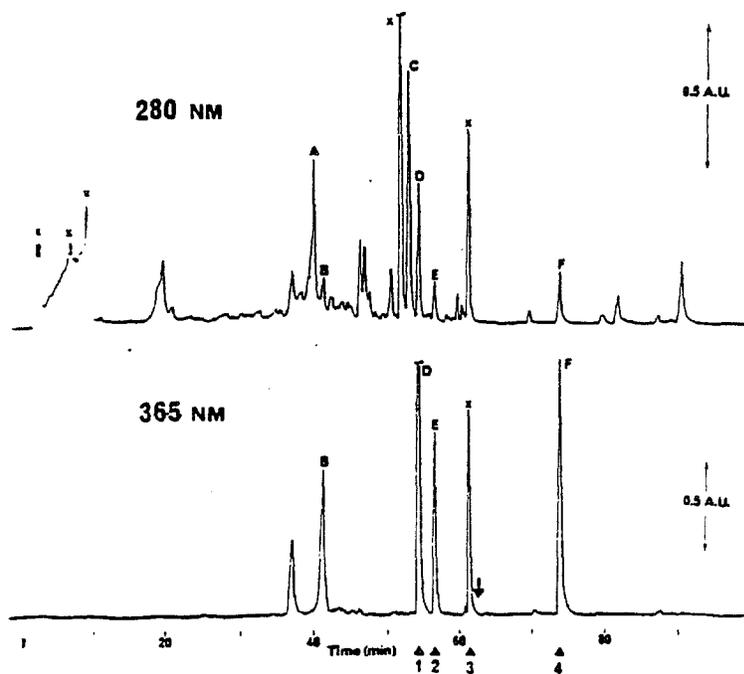


Figure 1.

The 280 and 365 nm chromatograms of one-half of the extract of 620 ml of pooled blood from psoriatic patients receiving Ro 10-9359. The retention times of reference Ro 13-7652 (1), Ro 10-1670 (2), retinol (3), and Ro 10-9359 (4) are indicated on the bottom of the 365 nm chromatogram. The peaks labeled "x" were also observed in the chromatogram of control blood extract. Gradient elution was used from 0 to 62 min (see arrow), followed by isocratic elution. The HPLC conditions are described in the experimental section.

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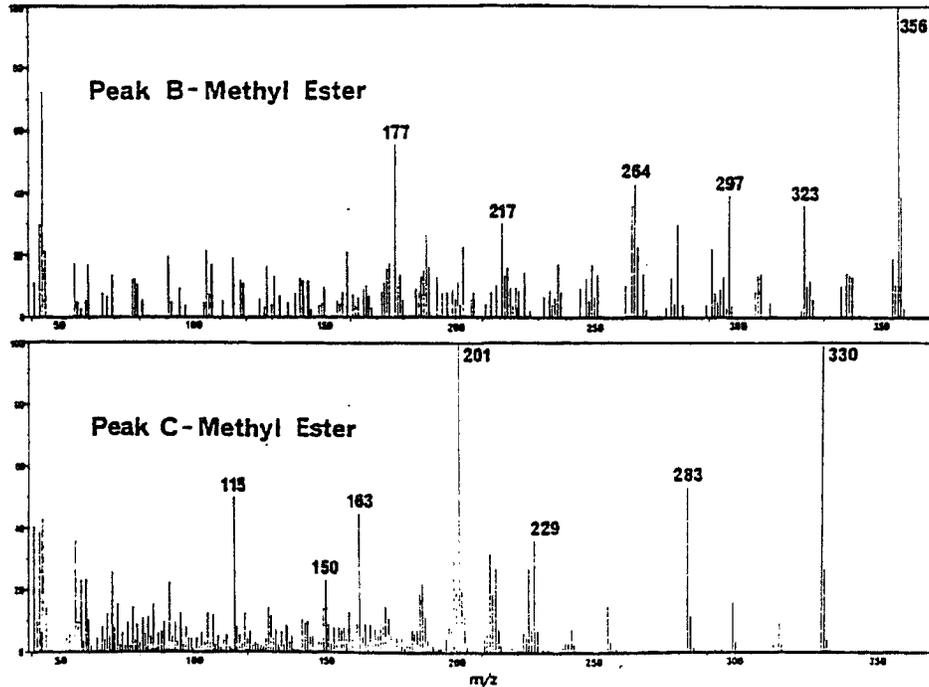


Figure 2.

Mass spectra of the methyl esters of peak B and peak C. The electron impact spectra were obtained on a VG ZAB-1F mass spectrometer at 70 eV.