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In vivo and in vitro metabolism of the new anticancer drug bisantrene

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Summary. *The metabolism of bisantrene, a new anthracene anticancer agent active in the treatment of disseminated breast cancer, was studied in vitro using rat liver S9 preparations and in vivo in patients receiving the drug as treatment for their cancers. ^{14}C-ring labeled bisantrene (248 mCi/40 mg) plus cold bisantrene were administered IV to cancer patients (260–340 mg/m^2). Fractional urine samples were collected at various time intervals up to 120 h after drug administration and analyzed by HPLC. The percent of total ^{14}C excreted as unchanged parent drug per ml urine ranged from 37 to 79% in the 0 to 24 h samples. The remainder of the radioactivity appeared chromatographically just prior to the bisantrene peak, indicating that compounds more polar than the parent were present as transformation products. Metabolism of bisantrene was also studied in vitro under oxic (O_2) and hypoxic (N_2) conditions, using commercially available Aroclor 1254 induced rat liver S9 preparations. Following N_2 incubation at 37° C for 1 h there was no evidence of metabolism, whereas there was more than 50% decrease in parent drug within 1 h following O_2 incubation in the presence of NADPH generating system, suggesting that the metabolic process involves an oxidative reduction. HPLC chromatogram profiles of the mixtures exposed to the activated S9 system indicated that there were at least 3 polar metabolites. In vitro human tumor clonogenic assay showed that the biological activity of bisantrene decreased greater than 4-fold when the drug was incubated with S9 preparations in the presence of NADPH and O_2, indicating that the transformation process leads to relatively inactive bisantrene metabolites.*

Introduction

Bisantrene, a new anthracene derivative, has shown significant antitumor activity against a wide variety of animal tumors [6] and in phase I and II clinical trials [2, 15, 16, 18]. Initial pharmacokinetic studies in patients have shown that bisantrene undergoes tri-exponential decay in plasma, with a prolonged terminal-phase half-life of approximately 30 h and greater than 95% binding to plasma proteins [4, 7, 11]. Although one study in animals has suggested no significant biotransformation of bisantrene [17], little information is available concerning its potential metabolism in patients [12].

In the present study we evaluated bisantrene metabolism in cancer patients in vivo and in rat liver S-9 preparations in vitro.

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Materials and methods

Drugs. Unlabeled and ^{14}C-ring-labeled bisantrene were provided for these studies by Lederle Laboratory, American Cyanamid Co., Pearl River, NY. The radiolabeled compound (specific activity = 6.2 μCi/mg) contained two ^{14}C atoms in the 9,10 positions, as shown in Fig. 1. The radiopurity was ≥ 95% as determined by HPLC.

Chemicals. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and NADP were obtained from Sigma Chemical Co. (St. Louis, Mo). Solvents used for HPLC were obtained from Burdick and Jackson Labs (Muskeogen, Mich) and were filtered and degassed prior to use. All other chemicals used were certified ACS grade (Fisher, Fair Lawn, NJ).

Microsomes. A commercially available microsome preparation was obtained from Litton Bionetics (Charleston, SC). The product consists of the S-9 fraction of homogenized rat livers, induced by Aroclor 1254. The preparation procedure used was that described by Ames et al. [5]. The protein content was specified as 21.1 mg/ml. Benzo(α)pyrene hydroxylase activity (used as a measure of enzyme activity) was 15.8 μmol hydroxybenzyprene per 20 min and mg protein. The stock S-9 fraction was stored at −80° C until use. An inactivated S-9 fraction was obtained by heat treatment at 56° C for 2 h just prior to use.

In vitro metabolism studies in human subjects. ^{14}C-ring-labeled bisantrene (248 μCi/40 mg) plus cold bisantrene were administered IV to cancer patients (260–340 mg/m^2). Fractional urine samples were collected at varying time intervals up to 120 h after drug administration and analyzed by HPLC as reported previously [11, 13]. Briefly, samples were extracted with Bond-Elut cartridges (Analytichem International, Harbor City, Calif). Aliquots of the extracts were injected for analysis of metabolic profiles by HPLC. The HPLC mobile phase was

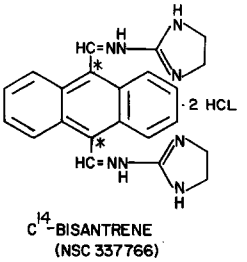

Fig. 1. Chemical structure of ^{14}C-bisantrene. *Asterisks* indicate ^{14}C atoms in positions 9, 10

comprised of acetonitrile-0.2 *M* ammonium acetate pH 4.0 (27 : 73) at a flow rate of 2.0 ml/min. A Waters Associates μBondapak C_{18} reversed-phase column was used for all analyses. Fractions of the HPLC effluent were collected every 30 s in liquid scintillation vials. Aquasol 10 ml was added to each vial and the radioactivities were counted using a Beckman LS-230 liquid scintillation counter.

Mass spectrometry. Fractions of the HPLC effluent from urine extracts were analyzed by solid-probe mass spectrometry to determine the structure of proposed metabolites by using a Finnigan Model 3300 mass spectrometer coupled to a model Incos data system (Finnigan Instruments, Sunnyvale, Calif). Selected ion monitoring was used to enhance sensitivity after a complete spectrum had been obtained from the parent compound. The characteristic ions at m/e 84, 111, 201, 228, 313, and 398 were used for both parent and metabolite confirmation.

In vitro drug stability studies. The in vitro stability of bisantrene in human urine was studied by adding ^{14}C-ring-labeled bisantrene to control human urine (2 μg/ml) and incubating at 37° C. Urine aliquots were assayed for bisantrene concentration at time intervals up to 24 h, and fractions of the HPLC effluent were counted for radioactivity as described above.

In vitro metabolism studies with rat liver S-9 preparations. The metabolism of bisantrene was studied in vitro using the commercially available rat liver S-9 preparations. The incubation mixture contained 0.25 ml S-9 (5 mg protein), 0.2 ml freshly prepared NADPH-generating system (1.3 μmol NADP, 9.8 μmol glucose-6-phosphate, 10 μmol $MgCl_2$, 2 U glucose-6-phosphate dehydrogenase in 0.05 *M* Tris-0.15 *M* KCl buffer, pH 7.4), 0.1 ml bisantrene, and Tris-KCl buffer. The total volume of the reaction mixture was 1.0 ml. Oxic and hypoxic conditions were achieved by stoppering tubes and pregassing reaction mixtures on ice for 5 min under a continuous flow of pure nitrogen or oxygen. The incubation tubes were then warmed at 37° C. After 1-h incubation at 37° C the reaction was stopped by adding 2 ml cold methanol, and the samples were mixed rapidly for 60 s and centrifuged at 3,000 rpm for 5 min at 4° C. The supernatant was injected directly for analysis of metabolic profiles by HPLC as previously described.

Human tumor clonogenic assay. A human tumor clonogenic assay [1, 8, 14] was used to study the effect of the S-9 fraction incubate on the biological activity of bisantrene against two human cell lines described below. The S-9 preparation was quickly thawed at 37° C, and diluted with cold 0.05 *M* Tris-0.15 *M* KCl buffer, pH 7.4 to a final concentration of 5 mg/ml protein. To assure sterility, the diluted S-9 preparations were filtered through a 0.45 μ*M* filter (Millex-HA, Millipore). The incubation mixture contained 1 ml filtered S-9 preparation (5 mg protein), 0.2 ml freshly prepared NADPH-generating system (1.3 μmol NADP, 9.8 μmol glucose-6-phosphate, 10 μmol $MgCl_2$, 2 U glucose-6-phosphate dehydrogenase in 0.05 *M* Tris-0.15 *M* KCl buffer, pH 7.4), 0.2 ml bisantrene (final concentrations of 0.01, 0.1, 1.0 and 10.0 μg/ml), 0.5 ml single-cell suspension and medium to give a final volume of 2 ml. After incubation at 37° C for 1 h the samples were centrifuged at 1,250 rpm for 10 min. The supernatant was injected directly for analysis of metabolic profiles by HPLC as described. The cell pellets were washed twice and plated on 35 × 10 mm petri dishes. Plates were incubated at 37° C, in a humidified atmosphere containing 7% CO_2 for 10–14 days. Plates with at least 30 colonies of 60 diameter were counted using an automated image analysis system (Bausch and Lomb Omicon FAS II).

Human tumor cell lines. The human myeloma cell lines 8226 was obtained from American Type Culture Collection (Rockville, Md). The cells were grown in a suspension in RPMI-1640 with 10% FCS and fed daily. Sixty thousand cells were seeded per plate.

The human breast cell line T47-D was kindly provided by Dr Oliver Alabaster (George Washington University Medical Center, Washington, DC). The cells were grown as a monolayer in McCoy's 5A with 10% FCS and subcultured every 7 days with trypsin-EDTA (10 x, Gibco, Grand Island, NY). Twenty thousand cells were seeded per plate.

Results

The urinary excretion profiles of bisantrene at varying time intervals up to 120 h after administration in eight cancer patients, as quantitated by total ^{14}C-radiolabeled counts or by HPLC, is shown in Table 1. Cumulative recoveries in urine were low, averaging < 15% of the administered dose over a period of 120 h, with most of the drug excreted in the first day after dosing. There were significant differences ($P < 0.05$) between the individual and cumulative means of the ^{14}C and HPLC data.

As shown in Fig. 2, the radiopurity of the standard ^{14}C-bisantrene used in these studies was > 95% and no detectable decomposition was observed after incubation of the drug in human urine at 37° C for up to 24 h.

The HPLC chromatogram profile of total ^{14}C-radioactivity in patient urine was studied at time intervals of 4–24 h after bisantrene administration. A sample chromatogram appears in Fig. 3. The radioactivity appearing in the bisantrene HPLC peaks ranged from 37% to 79% of the total ^{14}C-radioactivity per milliliter of urine (Table 2). The remainder of the radioactivity appeared chromatographically just prior to the bisantrene HPLC peak (Fig. 3).

Table 3 shows the results of the in vitro metabolism studies with rat liver S-9 preparations. Incubation with the NADPH-generating system had no effect on bisantrene's chemical stability. Incubation with S-9 preparations with or without the NADPH-generating system (active or heat

Table 1. Urinary excretion of bisantrene (^{14}C and HPLC) in eight patients, suggesting urinary metabolite(s)

Interval after dosing (h)	Mean percent of dose excreted		
	Total ^{14}C	HPLC	Wilcoxon P-value
0– 4	6.74 ± 2.441	4.99 ± 1.533	0.021
4– 8	3.02 ± 2.273	1.78 ± 1.302	0.018
8– 24	1.61 ± 0.463	0.81 ± 0.338	0.018
24– 48	0.81 ± 0.259	0.39 ± 0.209	0.012
48– 72	0.47 ± 0.345	0.13 ± 0.076	0.012
72– 96	0.34 ± 0.109	0.09 ± 0.073	0.012
96–120	0.29 ± 0.134	0.08 ± 0.064	0.043
Cumulative mean ± SD	12.94 ± 3.08	8.13 ± 2.56	0.018

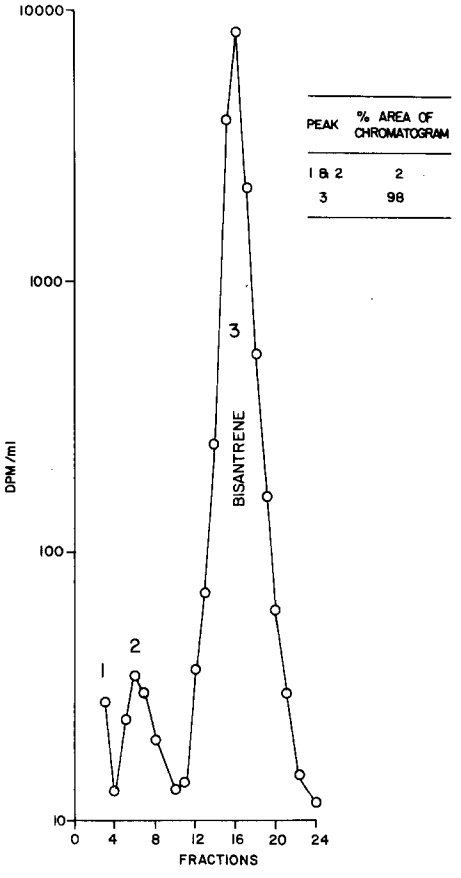

Fig. 2. HPLC chromatogram of ^{14}C-bisantrene added to human urine (2 μg/ml) after 24 h at 37° C

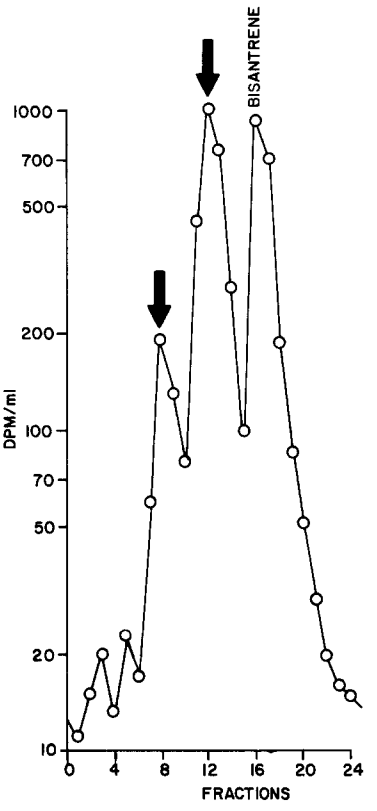

Fig. 3. HPLC chromatogram of ^{14}C-bisantrene in an urine sample collected from 8- to 24 h after administration

Table 2. Percent of total ^{14}C-radioactivity recovered from patient urine samples after bisantrene administration

Patient	Percent of HPLC chromatogram areas					
	0–4 h		4–8 h		8–24 h	
	Bisantrene	Metabolites[a]	Bisantrene	Metabolites[a]	Bisantrene	Metabolites[a]
1	79.1	18.5	–	–	57.3[b]	38.6[b]
2	79.1	20.2	67.1	27.9	74.1	23.6
3	70.4	25.6	37.7	54.5	37.2	54.5

[a] Potential metabolites of bisantrene
[b] 4- to 24 h sample

inactivated) resulted in an approximately 10% loss in bisantrene concentration with incubation under N_2. However, there was a statistically significant decrease in parent drug concentration to 46.8% of control values within 1 h when active S-9 fraction and NADPH-generating system were incubated together under oxic conditions. HPLC chromatogram profiles of the mixtures exposed to the activated S-9 system in the presence of oxygen showed the generation of three potential metabolites (Fig. 4) which were more polar than the parent, bisantrene.

Figures 5 and 6 show the effect of the rat liver S-9 fraction on the biological activity of bisantrene against two human cell lines. Incubation with the NADPH-generating system or S-9

Table 3. Effect of activated rat liver S-9 fraction on bisantrene (HPLC) in vitro (1-h incubation)

Incubation system[a]	Bisantrene concentration (percent of control, $\bar{X}$+SD)
NADPH (no S-9), O_2 or N_2	98.5 ± 1.75
Active S-9 (no NADPH), O_2 or N_2	89.1 ± 3.1
Inactive S-9 + NADPH, O_2 or N_2	89.7 ± 2.3
Active S-9 + NADPH, N_2	88.3 ± 2.8
Active S-9 + NADPH, O_2	46.8 ± 5.2

[a] See *Methods* for details

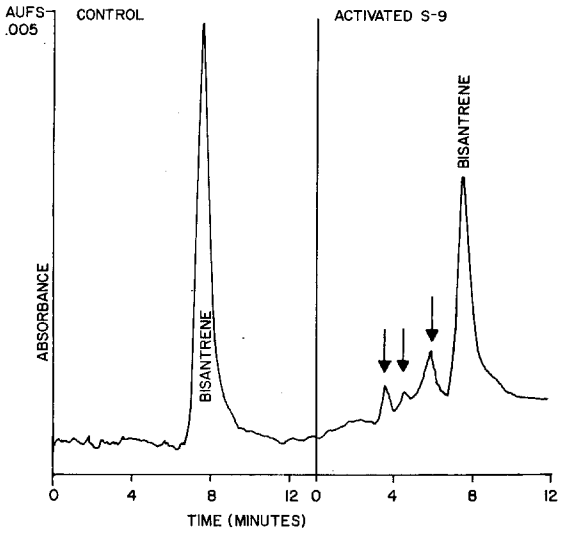

Fig. 4. HPLC chromatogram of bisantrene: presumed metabolite peaks generated by activated rat liver S-9 fraction

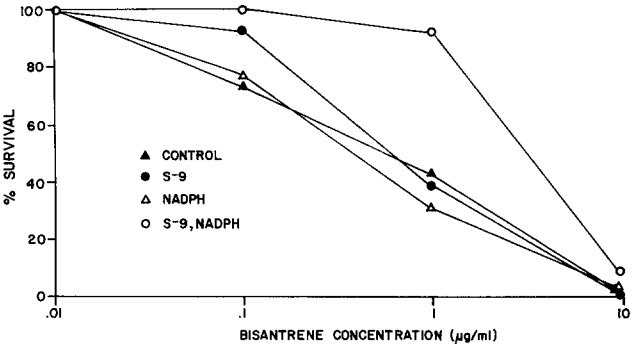

Fig. 6. Effect of rat liver S-9 fraction on the biological activity of bisantrene against human breast cell line T47-D

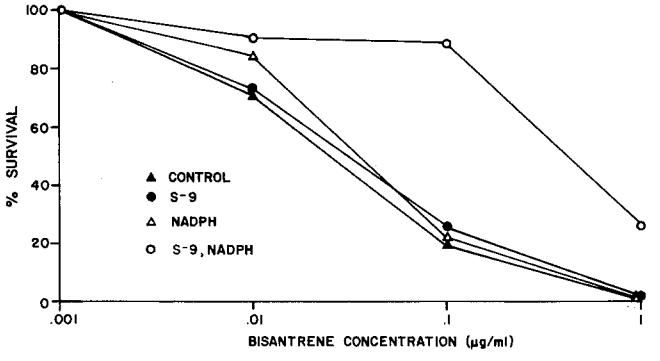

Fig. 5. Effect of rat liver S-9 fraction on the biological activity of bisantrene against human myeloma cell line 8226

alone had no significant effect on the biological activity of bisantrene. When incubated with activated S-9 system under oxic conditions there was more than a four-fold decrease in bisantrene activity at a concentration of 0.1 μg/ml (1-h incubation period) with respect to the inhibition of tumor colony-forming units from the human myeloma cell line 8226 (Fig. 5). Likewise, when the fully constituted S-9 NADPH system was incubated with the human breast cell line T47D under oxic conditions there was a three-fold reduction in bisantrene's inhibition of tumor-colony-forming units at a concentration of 1 μg/ml (Fig. 6).

Samples of bisantrene extracted from patient urine and purified by HPLC were analyzed by solid-probe mass spectrometry. Selected ion monitoring showed the presence of the molecular ion of bisantrene (M + 398) and other characteristic fragments at scans 64–65 (Fig. 7), thus confirming the presence of bisantrene in the patient urine. Some uneven volatilization of bisantrene is also shown in scans 60–61 of Fig. 7. This was probably due to some nonvolatile HPLC mobile phase constituents in the sample interfering with the volatilization of bisantrene in the sample cuvette. Proposed metabolites which eluted prior to bisantrene in the HPLC profile shown in Fig. 4 were also subjected to solid-probe-selected ion monitoring mass spectrometry (Fig. 8). These metabolites were produced from incubations with S-9 preparations.

Discussion

Wu and Nicolau [17] have reported that bisantrene does not undergo significant biological transformation in small or large laboratory animals (i.e., rats, dogs, and monkeys). The results of the in vitro rat liver and in vivo human studies described in this report appear to document extensive bisantrene metabolism. First, the differences in the total amount of bisantrene urinary excretion as measured by HPLC (unlabeled drug) and ^{14}C-radiolabeled studies indicate the possibility of drug metabolism and/or decomposition. The latter does not appear to be a primary clearance process, since bisantrene is stable in vitro in both human plasma and urine samples at 37° C for more than 24 h. Secondly, HPLC chromatogram profiles in urine samples obtained from 0 to 24 h in three patients after treatment with ^{14}C-bisantrene revealed the presence of at least two compounds (ranging from 21% to 63% of the total ^{14}C-radiolabeled counts per milliliter of urine) more polar than the parent. Thirdly, the results of our in vitro rat liver S-9 fraction studies with bisantrene indicate that its transformation to these more polar metabolites requires a full NADPH-generating system and molecular oxygen. This suggests that the metabolic process involves an oxidative-reductive process. The differences between our results and those of Wu and Nicolau [17] could be explained partly by differences in analytical approach (they used gas-liquid and thin-layer chromatography) and/or experimental species. Nevertheless, there appears to be strong evidence of in vivo bisantrene metabolism in cancer patients.

The effect of S-9 incubation of the biological activity of bisantrene was tested against two human cell lines using a human tumor clonogenic assays. The fact that the biological activity of bisantrene decreased when the drug was incubated with activated S-9 system suggests that the transformation process leads to relatively inactive metabolites. Characterization of these potential metabolites is currently under investi-

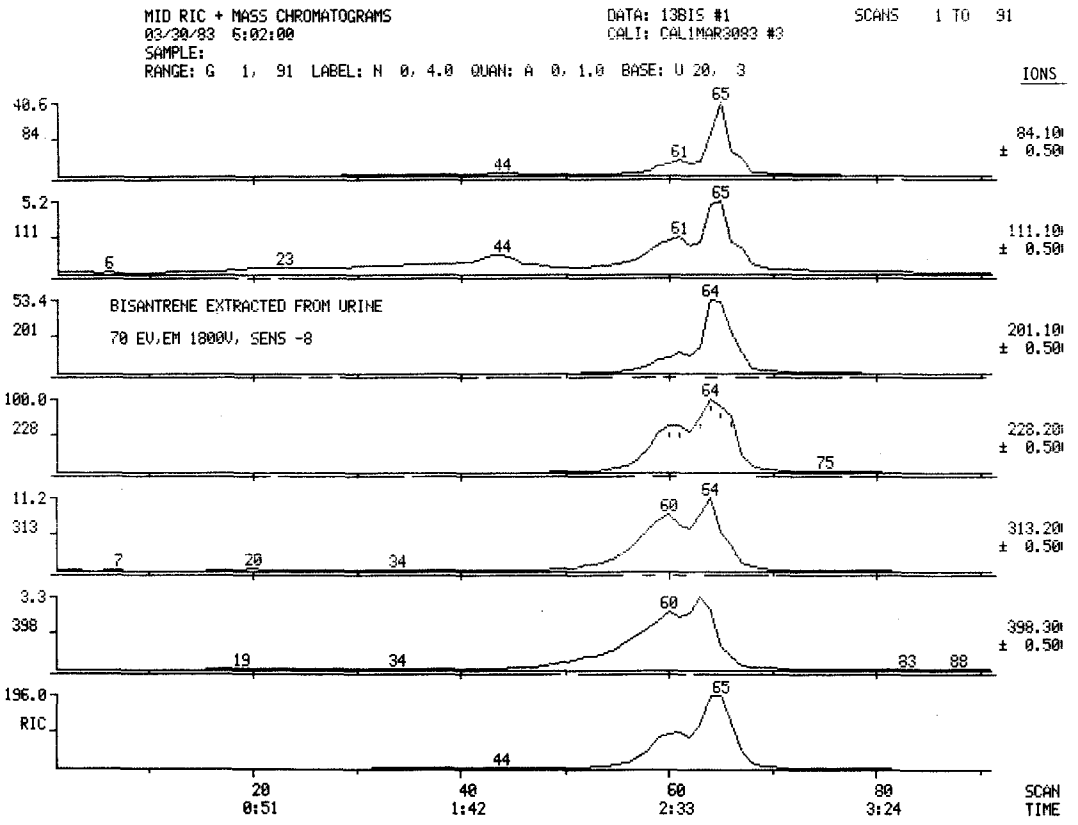

Fig. 7. Selected ion monitoring mass spectrometry of bisantrene from a patients urine. The molecular ion at m/e 398 is shown, as are characteristic ions at 313, 228, 201, 111 and 84

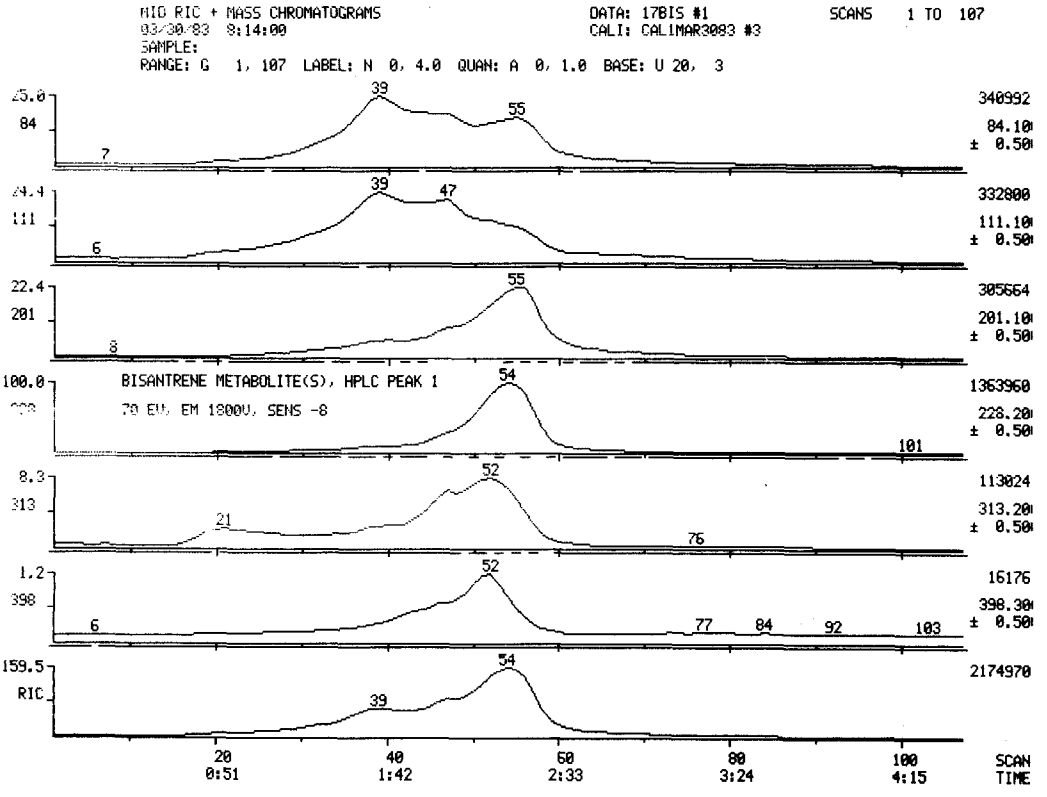

Fig. 8. Selected ion monitoring mass spectrometry of proposed metabolite isolated from HPLC peak

gation by mass spectrometry techniques. The most nonpolar proposed metabolite, eluting just prior to bisantrene (scan 39, Fig. 8), was found to be more volatile than the parent compound. The fragments of greatest abundance for the proposed metabolite were m/e 84 and 111. These fragments are derived from the imidazole rings and side chains. The fact that this metabolite is more volatile than bisantrene suggests that it has a lower molecular weight than the parent. This metabolite also yields a small amount of the fragments at 201 and 313. These are associated with the anthracene moiety and would not be present if the anthracene had been metabolized. More confirmation is necessary on additional samples to confirm this finding.

On the basis of the results of prior pharmacokinetic studies of bisantrene in patients [3, 4, 9, 10], it is reasonable to suspect that hepatic metabolism may be a major elimination route of this drug. Urinary and fecal excretion measured by HPLC (urinary) or ^{14}C-bisantrene equivalents (fecal) averaged $< 7\%$ and 17%, respectively [3, 4,]. The relatively low renal clearance of bisantrene has been confirmed by other investigators [9, 10]. Unfortunately, insufficient clinical data on bisantrene exist to indicate whether hepatic dysfunction or hyperbilirubinemia is associated with reduced plasma and whole-body clearance rates and/or increased bone marrow toxicity. Thus, the clinical significance of bisantrene metabolism is uncertain and awaits further laboratory and clinical investigation.

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