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Metabolism and elimination of rhodamine 123 in the rat*

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Summary. Little is known of the pharmacology of rhodamine 123 (RH-123), an agent reported to have carcinoma-selective experimental antitumor activity. Accordingly, using a high-performance liquid chromatographic assay system with fluorescence detection, we examined the plasma decay and the biliary and urinary elimination of parent drug and metabolites in female Sprague-Dawley rats receiving RH-123 at an intravenous dose (5 mg/kg) equivalent to the therapeutic dose used in murine tumor models. Following drug administration to unconscious animals, plasma levels of drug-associated fluorescence fell in a triphasic manner ($t_{1/2\alpha}$, 15 min; $t_{1/2\beta}$, 1 h; $t_{1/2\gamma}$, 4.7 h). In plasma, unchanged drug predominated but lower levels of the deacylated metabolite rhodamine 110 (RH-110) and two unknowns were also detectable throughout the study. Drug fluorescence was recovered extensively in both urine and bile. In unconscious animals with ureteral cannulae, urinary excretion (11.4% of the dose in 6 h) occurred predominantly as unchanged RH-123 (97% of the total), with low levels of RH-110 (2.4%) and two unknowns (<0.6% combined) also being present. Similarly dosed conscious animals (without surgical intervention) housed in metabolic cages showed a comparable pattern of urinary excretion, with 11.9% of the drug dose being recovered in 6 h and 21.9%, by 48 h. Biliary drug elimination accounted for 8% of the delivered dose in 6 h in unconscious animals and for 11% by 36 h in conscious animals fitted with biliary cannulae. In contrast to urinary excretion, in which unchanged drug predominated, only 50% of the fluorescence recovered in bile was attributable to RH-123. The remainder was due to a number of products that were detectable throughout the study. Of these, one present at significant levels was identified as a glucuronide conjugate of RH-123, based on the liberation of parent drug when the purified metabolite was incubated with β-glucuronidase or hydrolyzed with 1 N hydrochloric acid. Further studies with a radiolabeled form of RH-123 are necessary to establish the identity of the remaining unknowns disclosed in this work.

Introduction

The permeant cationic dye rhodamine 123 (RH-123, Fig. 1) currently enjoys use in fluorescence microscopy as a mitochondrial-specific vital stain [9]. In recent years this compound has received additional attention based on its reported carcinoma-selective antitumor activity. In this regard, RH-123 has been shown to alter markedly the clonal growth of cultured carcinoma cell lines, whereas it has little effect on other, nontumorigenic epithelial cells [2]. Likewise, RH-123 produced significant extension of the survival of mice bearing Ehrlich ascites or MB49 bladder carcinomas but was ineffective against murine L1210 and P388 leukemias and the B16 melanoma tumor-model systems [3]. Furthermore, its activity against rat prostatic tumor [17], murine renal adenocarcinoma [7], and other epithelially derived tumor cell lines [6] has been documented; moreover, the use of RH-123 as a photosensitizer

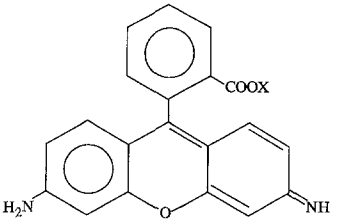

Fig. 1. Structures of RH-123 (X = CH_3) and its deacylated metabolite RH-110 (X = H)

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in the argon-laser therapy of tumors and as an enhancer in hyperthermic cancer treatment are being explored [4, 10, 16]. The precise cytotoxic mechanism of this agent remains unclear. However, a number of studies indicate that certain rhodamine dyes are selectively retained in the mitochondria of carcinoma cells and that their presence in these cells results in disruptions of ATP synthesis and mitochondrial respiration [3, 5, 8, 11–15, 17]. Thus, the carcinoma-selective antitumor effects of RH-123 may be associated with a non-nuclear target site of action. This idea is intriguing since currently used drugs directed against DNA show little efficacy against these neoplasias.

Our interest in RH-123 derives from considerations of its possible utility in the intravesical therapy of superficial bladder carcinoma. Using a sensitive high-performance liquid chromatography (HPLC) assay system developed at our institution [19], we have previously shown in a rat intravesical model that little drug is absorbed and systemically circulated when RH-123 is instilled and maintained for several hours in the urinary bladder; systemic plasma levels of RH-123 through 9 h following the intravesical administration of a 5-mg/kg drug dose were at all times below those measured for the long terminal phase when the same drug dose was given intravenously [18, 19]. Consequently, the intravesical route of drug administration, which enables tumor to be bathed by a high concentration of cytotoxic agent, is unlikely to be associated with significant RH-123 systemic toxicity. As part of our work, we also identified the deacylated product rhodamine 110 (RH-110, Fig. 1) as a minor plasma metabolite of RH-123 [18]. Little else is known of the pharmacology of RH-123, except for a report describing the presence, without quantitation, of RH-123 and RH-110 in an extract derived from a human ML tumor excised from the flank of a nude mouse 24 h after the intraperitoneal administration of a 10-mg/kg dose of drug [1]. We have now extended our analytical capability to study the disappearance of RH-123 from plasma as well as the biliary and urinary elimination of parent drug and metabolites in conscious and unconscious rats following the intravenous administration of RH-123 at a dose equivalent to the therapeutic dose used in murine tumor models. This report thus represents the initial description of the pharmacokinetics of RH-123 in an animal system.

Materials and methods

Chemicals

RH-123 (laser grade), RH-110, and crystal violet were purchased as hydrochloride salts from Eastman Kodak Co. (Rochester, N.Y.). The RH-123, which was about 96% pure when received, was further purified by open-column chromatography on silicic acid (Biosil A; Bio-Rad Laboratories, Richmond, Calif.) with chloroform-methanol (6%) elution prior to its use in animals. The resulting material assayed at >99.8% purity by reversed-phase HPLC with simultaneous fluorescence and ultraviolet-absorption detection.

[1] NCI Diluent 12, a 1 : 1 (vol/vol) mixture of Cremophor EL (polyethoxylated castor oil) and ethyl alcohol, was kindly provided by the Pharmaceutical Resources Branch, National Cancer Institute

Sample collection

Unconscious animals. Female Sprague-Dawley rats (250–300 g) that had been anesthetized with sodium pentobarbital (60 mg/kg) were surgically fitted with catheters in the external jugular vein (for drug administration) and carotid artery (for blood sampling), with cannulation of either the bile duct or the ureters for the continuous collection of bile or urine. Animals remained unconscious for the duration of the study (6 h), with body temperatures being maintained on isothermal heating pads (Braintree Scientific, Braintree, Mass.). RH-123 (5 mg/kg), formulated in 20% NCI Diluent 12[1]-80% saline, was given as an intravenous bolus over a 15-s period. The catheter was cleared with saline and the saline flow was maintained to provide blood volume replacement. At predetermined times (from 5 to 360 min) after dosing, blood samples (50–400 µl) were collected and centrifuged and the resulting plasma was frozen at –70° C pending analysis. Biliary and urinary flow from the respective cannulae were collected in preweighed plastic microcentrifuge tubes (1.5-ml capacity) at hourly intervals and the samples were immediately frozen and stored at –70° C.

Conscious animals. In one set of studies, female Sprague-Dawley rats (250–300 g) under momentary methoxyfluorane anesthesia were given a single intravenous dose of RH-123 (5 mg/kg) via the tail vein. Animals were then returned to individual metabolic cages. At preselected times (6, 12, 24, 36, and 48 h), total urine and fecal collections were harvested, quick-frozen, and stored at –70° C.

In another study, rats under methoxyfluorane anesthesia were fitted with bile-duct cannulae and femoral vein catheters for bile collection and drug dosing, respectively. The tubes were brought to the exterior at the tail, where they were conducted through the cage wall via a coiled spring secured to the cage wall at one end and surgically attached to the base of the tail at the other. Animals were allowed to recover overnight from surgery before receiving drug and were allowed free access to food and water throughout the study. The patency of the venous line was maintained by the continuous infusion of 0.8% sterile sodium chloride (10 ml/24 h). RH-123 (5 mg/kg) was given as a bolus over 15 s and the venous catheter was cleared with saline. Cumulative bile samples were collected at various times (3–36 h) and then frozen at –70° C pending analysis.

Sample preparation

Plasma samples were extracted as follows: C_{18} Sep-Pak minicolumns (Waters Associates, Milford, Mass.) were sequentially equilibrated with methanol, methanol:water (1 : 1, v/v), and phosphate buffer (pH 8.5). The plasma samples were then applied. The minicolumns were washed with phosphate buffer (pH 8.5) and extracted with acidified methanol (0.1% HCl). The extracts were evaporated to dryness under a stream of dry nitrogen (37° C water bath) and stored at –70° C. For analysis, samples were reconstituted in methanol (100–200 µl), with crystal violet being added as the internal standard. Bile and urine samples were analyzed by direct injection onto the HPLC column without prior extraction.

Separation and quantitation

Samples were analyzed by reversed-phase HPLC according to a previously described scheme [19]. In brief, separation was accomplished on a phenyl Radial-Pak radial compression column (Waters Associates) using an acetonitrile-ammonium formate buffer (0.05 M, pH 4.0) elution. Signals were monitored by flow fluorometry (Schoeffel model FS-970; Kratos Schoeffel Instruments, Ramsey, N.J.) at an excitation wavelength of 485 nm and using a 550-nm barrier filter. RH-123 and RH-110 signals were identified by their retention times relative to authentic standards and by their ultraviolet-visible absorption spectra. Plasma samples were quantified by reference to standard curves constructed for RH-123 that had been added to blank plasma and processed as above, with appropriate corrections for the extraction efficiency. Urine and bile samples were

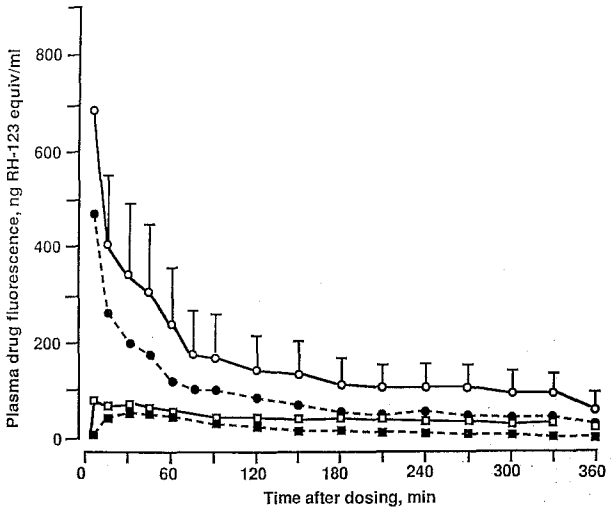

Fig. 2. Plasma concentrations (ng RH-123 equivalents/ml) of parent drug and metabolites over 6 h following the administration of a single intravenous bolus dose of 5 mg/kg RH-123 to unconscious female Sprague-Dawley rats (n = 10). ○——○, total drug fluorescence ± SD; ●- - - -●, RH-123; □——□, RH-110; ■------■, unknown plasma metabolites P1 and P2 combined

Table 1. Cumulative urinary fluorescence recovery of parent drug and metabolites from conscious (n = 6) and unconscious (n = 6) female Sprague-Dawley rats following a single intravenous bolus dose of 5 mg/kg RH-123

Time (h)	Cumulative recovery of delivered dose (mean % ± SD)	Percent recovered as		
		RH-123	RH-110	Unknowns[a]
Unconscious animals[b]:				
1	1.6 ±0.8	95.3	2.4	2.3
2	5.12±1.94	97.1	1.4	1.5
3	7.51±2.66	96.6	1.8	1.6
4	9.3 ±2.88	96.1	2.3	1.6
5	10.75±3.26	94.3	3.7	2
6	11.41±3.39	91	6.1	2.9
Conscious animals[c]:				
6	11.91±0.82	95.1	4.7	0.2
12	16.75±1.97	78.6	19.8	1.6
24	20.19±2.02	70.5	27.7	1.8
36	21.19±2.24	70.8	26.9	2.3
48	21.87±2.37	61.3	37.6	1.1

[a] Based on the amounts of urinary signals U1 and U2 combined
[b] Surgically fitted with bilateral ureteral cannulae for the continuous collection of urine
[c] Without surgical intervention; animals housed in metabolic cages

quantified relative to directly injected standards prepared in methanol. The detector response for signals other than RH-123 was expressed in nanogram equivalents of RH-123 and did not take into account possible differences in fluorescence quantum efficiencies for these materials relative to RH-123.

Isolation of unknowns

After HPLC analysis of the individual timed bile samples, the remaining materials were combined in phosphate buffer (pH 8.5) and extracted with chloroform: 1-propanol (3 : 1, v/v). This process removed RH-123 and RH-110 from the other fluorescent materials that remained in the aqueous phase. The latter were extracted with C_{18} Sep-Paks, eluted with methanol, and separated by repetitive HPLC injections, as before, but using a microfractionater (Gilson Electronics, Inc., Middleton, Wis.) to collect the appropriate fractions for further study. The individual fractions were recovered by extraction with C_{18} Sep-Paks in the usual manner, with the process being repeated until homogeneity was achieved [single-peak material by both fluorescence (485-nm excitation; 550-nm cutoff filter) and ultraviolet absorption (254 nm; Waters Associates model 440 absorption detector)]. Homogeneity of the individual fractions was further determined by thin-layer chromatography (TLC) on glass-backed silica gel GF plates (250-nm layer; Analtech Inc., Newark, Del.) using a developing system of chloroform: methanol:water (100:40:4, by vol.). Purified samples were reconstituted in methanol for recording of their ultraviolet-visible spectra (Lambda-3B spectrophotometer; Perkin-Elmer, Norwalk, Conn.). Two of the unknowns were obtained in sufficient quantity for further study. These were subjected to room-temperature acid hydrolysis (1 N HCL) and incubation with β-glucuronidase (Sigma, St. Louis, Mo.) at 37°C in pH 5.0 buffer, with HPLC monitoring.

Results

The disappearance of RH-123 and the appearance of drug metabolites in plasma during the 6 h following the intravenous administration of a 5-mg/kg dose of drug to unconscious animals can be seen in Fig. 2. Levels of both parent drug and total drug-associated fluorescence fell essentially in parallel in an apparent triphasic manner, with initial half-lives of about 15 min and terminal half-lives of about 4.7 h (based on 6 h observation). An intermediate redistribution phase with a half-life of 1 h was also apparent. Mean total fluorescence clearance from the plasma (dose/AUC) was 113.5 ± 48 ml min^{-1} kg^{-1} for the period from 0 to 6 h. Unchanged RH-123, with a retention time of 9.52 min, was the predominant fluorescent signal detectable at all times. Three other signals were also seen, the largest of these being attributable to RH-110 (retention time, 4.99 min). The remaining two signals were both quite minor. These materials, designated unknown P1 and P2 (retention time, 3.17 and 8.07 min, respectively), have not yet been characterized.

Table 1 shows the recovery of parent drug and metabolites in the urine of rats receiving 5 mg/kg RH-123. In both conscious and unconscious animals, the recovery of drug fluorescence by this route over the first 6 h was primarily attributable to unchanged drug (>94% of the total), with the balance being due almost exclusively to RH-110. From animals housed in metabolic cages, the proportion of unchanged drug in the urine began to fall significantly after 6 h, reaching a low of 61% of the recovered drug fluorescence over the period between 36 and 48 h. The decline in RH-123 levels in the urine of these animals was accompanied by a commensurate increase in the recovery of RH-110. Very low levels of two other products (unknowns U1 and U2) accounted for the remainder of the recovered drug fluorescence; these materials exhibited retention times identical with the minor plasma metabolite unknowns P1 and P2. Comparison of the recovery of urinary drug fluorescence from unconscious surgically manipulated animals with that from animals not subjected to surgical intervention that were maintained in metabolic cages showed close agreement (11.4% vs 11.9% through 6 h).

Table 2. Cumulative biliary fluorescence recovery of parent drug and metabolites from conscious ($n = 6$) and unconscious ($n = 6$) female Sprague-Dawley rats following a single intravenous bolus dose of 5 mg/kg RH-123

Time (h)	Cumulative recovery of delivered dose (mean % ± SD)	Percent recovered as				
		RH-123	RH-123 glucuronide	Unknown B1	Unknown B3	Unknown B4
Unconscious animals[a]:						
1	5.15 ± 0.51	48.6	30.6	5	9.1	6.7
2	6.79 ± 0.7	46.8	25.7	6.6	12.4	8.5
3	7.36 ± 0.89	47.2	22.3	4.4	14	11.9
4	7.69 ± 1.07	55.5	14.9	4	14.1	11.5
5	7.89 ± 1.18	54.7	13.3	5.1	15.3	11.6
6	8.05 ± 1.25	53.8	15.6	3	19.4	9.7
Conscious animals[b]:						
3	6.96 ± 1.22	55	32.3	4.1	3.5	5.2
6	8.73 ± 1.21	33.6	37.9	7.2	7.2	11.7
12	9.65 ± 1.3	34.6	38.2	6	9.2	12
24	10.31 ± 1.45	30.2	36.7	4.1	16.3	11.1
30	10.5 ± 1.5	38.6	25.7	5.4	16.8	11.5
36	10.77 ± 1.65	33.9	29	8	18.1	11

[a] Surgically fitted with bile-duct cannulae for the continuous collection of bile

[b] Surgically fitted with bile-duct cannulae, as above, but tethered via a coiled spring housing for the tail and the exteriorized cannula; animals were free to move about and had access to food and water ad libitum throughout the study

For the animals in metabolic cages, the overall urinary recovery of drug fluorescence was 22% in 48 h. The rate and half-life of the total urinary drug-fluorescence elimination was calculated from a semi-logarithmic plot of the percentage of the total recovered fluorescence remaining to be eliminated versus time. For the conscious animals (0–48 h), the rate constant was $0.127 \pm 0.024\ h^{-1}$, with a half-life of 5.67 ± 1.23 h.

Despite the relative simplicity of the RH-123 metabolite pattern in plasma and urine, biliary analysis revealed a more complex picture (Table 2). As in plasma and urine, unchanged drug was the predominant fluorescent signal detected in the bile of animals receiving 5 mg/kg RH-123. In unconscious animals, the amount of unchanged drug in the bile remained constant (~50% of the total drug fluorescence) at each hourly cumulative sampling time through 6 h. This value was comparable with the 55% of total drug fluorescence attributable to RH-123 in the initial 3-h sample of bile from conscious animals. Thereafter, in these animals, levels of RH-123 fell to only 34% of total drug fluorescence over the period from 30 to 36 h. Remarkably, little if any RH-110 was detected in either set of bile samples. However, four other fluorescent products were detected at levels sufficient to be quantified, and several additional trace signals were also seen. These signals were found throughout the studies in both conscious and unconscious animals, their combined levels rising from 15% to 32% total recovered fluorescence in the anesthetized animals (through 6 h) and from 13% to 36% in the conscious animals (through 36 h). Total recovery of drug fluorescence was comparable in the unconscious vs conscious groups from 0 to 6 h (8.1% vs 8.7%), with the latter group providing a total recovery of 11% of the dose by 36 h. The rate of hepatobiliary total drug-fluorescence elimination in the conscious animals (0–36 h) was $0.0855 \pm 0.0136\ h^{-1}$, with a half-life of 8.27 ± 1.26 h.

The four measurable unknown biliary fluorescent signals, with HPLC retention times of 3.12, 4.08, 6.65, and 8.07 min, were designated unknowns B1, B2, B3, and B4, respectively. Of these, unknowns B2 and B3 represented a significantly greater abundance than the other two. Unknowns B1 and B4 appeared to be identical in retention times with the minor plasma metabolites P1 and P2 and the unknown urinary metabolites U1 and U2. Based on material recovered from the HPLC, the ultraviolet-visible absorption spectra of unknowns B1 and B4 have been recorded but, because of their very limited availability, no further work has yet been possible with these products.

The two principal unknown biliary fluorescent materials, B2 and B3, were obtained by repeat HPLC sampling, and each was shown to be homogeneous by TLC and by HPLC using both fluorescence and absorption detection. Incubation of unknown B2 with β-glucuronidase in pH 5.0 buffer at 37° C resulted in a time-dependent degradation of the substrate, with the appearance of RH-123, as monitored by HPLC. The emergence of RH-123 was also seen when unknown B2 was allowed to stand overnight in 1 *N* HCl at room temperature. The identity of the RH-123 derived from the incubation of unknown B2 with β-glucuronidase was confirmed by co-chromatography (HPLC, TLC) with authentic material, ultraviolet-visible spectrophotometry, and mass spectrometry (m/e 344). An authentic reference sample of RH-123 was shown to be unaltered after standing in pH 5.0 buffer at 37° C in the absence or presence of β-glucuronidase. Based on these several lines of evidence, unknown B2 was thus identified as RH-123 glucuronide. Attempts to obtain a direct parent-ion identification of this conjugate by fast atom bombardment-mass spectral (FAB-MS) analysis were unsuccessful, although a signal corresponding to RH-123 was seen as a degradation product.

Unknown B3 (TLC R_f, 0.52; chloroform: methanol:water, 60:40:4 by vol.) was unaffected by exposure to

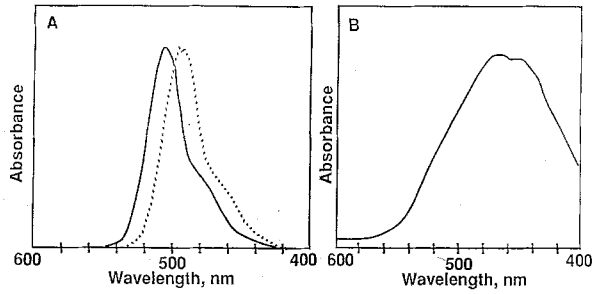

Fig. 3 A, B. Visible absorption spectral profiles determined in methanol. **A** Authentic RH-123 (*solid line;* λ max., 507 nm) and RH-110 (*dashed line;* λ max., 496 nm). **B** Unknown biliary metabolite B3 (λ max., 476, 455 nm)

β-glucuronidase under the conditions previously used with unknown B2. Attempts to obtain FAB-MS data for this material have not been successful. The visible absorption spectrum of unknown B3 is shown in Fig. 3B.

Discussion

The present study was undertaken in anticipation of the potential application of RH-123 in the intravesical therapy of superficial bladder carcinoma. In murine tumor-model systems, the effective dose of RH-123 has been reported to be 10 mg/kg [3]. In a previous study, the rat equivalent of this dose, 5 mg/kg, when instilled intravesically and allowed to remain in the bladder for 2 h, was shown to result in very low circulating plasma levels of parent drug and deacylated metabolite RH-110 [18]. To assess the potential significance of these drug levels, we examined the pharmacology of RH-123 when the same dose of drug was given systemically.

From the results presented herein, it can be seen that following intravenous bolus administration, plasma drug fluorescence shows a period of rapid distribution and a more gradual elimination phase. A possible redistribution phase with a half-life of approximately 1 h can be inferred from the change of slope of the RH-123 and total drug-fluorescence curves. Although plasma and urine studies suggest only limited biotransformation of RH-123, the analysis of bile shows an apparently greater extent of metabolism.

Drug fluorescence was excreted extensively in both urine and bile. Over 36 h following drug administration, almost one-third of the dose could be accounted for, with urinary elimination being favored over the biliary route by a factor of 2:1. In urine, in addition to unchanged drug, RH-110 was seen as the predominant yet minor metabolite. The appearance of RH-110 as a biotransformation product of RH-123 was anticipated based on the ubiquitous occurrence of plasma and tissue esterases capable of effecting this deacylation. It was thus surprising that little or no RH-110 was seen in the bile of rats receiving RH-123. Instead, bile was found to contain a number of fluorescent species, predominantly including a polar conjugate unambiguously shown to be an RH-123 glucuronide.

The unknowns B1, B3, and B4 are deemed to be drug-derived, based on their appearance in the bile of RH-123-treated animals and their absence in the bile of untreated animals. However, it is not certain at this time that all of these materials are indeed RH-123 metabolites. The visible absorption spectrum of unknown B3, as seen in Fig. 3B, is similar in profile to those of unknowns B1 and B4, although the individual absorption maxima are somewhat different from each other. Overall, the spectral characteristics of these signals do not resemble those of RH-123 or RH-110 (Fig. 3A) but appear to be more like those of the bile pigments bilirubin and biliverdin, although they clearly do not match the characteristics of these endogenous materials. In this regard, we previously showed that RH-123 has an affinity for red blood cells [18]. The possibility thus exists that some or all of these unknown signals may be due to protoporphyrin breakdown products, perhaps the result of drug-induced hemolysis. This point remains to be further examined. However, such a finding would not materially alter the general picture of RH-123 pharmacokinetics derived here, since these unknown materials contribute in only a very minor way to the plasma, urine, and early bile data based primarily on unchanged RH-123, RH-110, and RH-123 glucuronide.

In conclusion, the in vivo studies described herein show that RH-123 undergoes significant metabolic transformation, with parent drug and metabolites being fairly rapidly excreted in urine and, to a lesser extent, in bile. At the same time, the overall recovery of only 32% of the delivered drug fluorescence in 36 h would also indicate that RH-123 undergoes a reasonable degree of tissue sequestration. However, it is also possible that further excretion of the delivered dose might have taken place in the form of nonfluorescent metabolites, especially in the bile, where the greater number of signals seen could be indicative of increased hepatic biotransformation. The use of a radiolabeled form of RH-123, with parallel monitoring of drug fluorescence and radiolabel, is needed to address this question further and to determine the origin of the as-yet uncharacterized fluorescent signals seen in the plasma, urine, and, especially, the bile of treated animals.

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