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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC QUANTITATION OF RHODAMINES 123 AND 110 FROM TISSUES AND CULTURED CELLS

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SUMMARY

Rhodamine 123 is a fluorescent vital dye which has potential for therapeutic use in cancer treatment. The dye concentrates in mitochondria of normal and neoplastic cells but accumulates in and is toxic to neoplastic cells. When dye-treated cells are irradiated with blue laser light at 514 nm, mitochondrial injury or cell death results. Rhodamine concentration in cultured cells and tumor tissue was quantitated to correlate cell or tumor death with drug dose. A reversed-phase separation of rhodamine 123 was accomplished using a gradient of 0.05 *M* phosphate buffer pH 2.85 (mobile phase A) and acetonitrile (mobile phase B), 10–80% B in 15 min with a DuPont Golden Series C_8 column. Effluent was monitored with a fluorescence detector at 295 nm excitation and 520 nm emission. Stock rhodamine 123 contained approximately 6–8% of rhodamine 110, the parent compound, which eluted at 9.8 min whereas rhodamine 123 eluted at 11.7 min. Structural verification of both compounds by field desorption mass spectrometry was performed.

This is the first report of the chemical separation and quantitation of rhodamine 123 from cultured tumor cells or tumor tissue.

INTRODUCTION

The cationic lipophilic dye, rhodamine 123 (Rh123) has been used recently as a specific probe for the localization of mitochondria in the living cell[1], but has been used as a histologic stain for almost 50 years[2,3]. This compound and its cationic analogues enter the cell and are sequestered at or inside the mitochondria[2,4]. Many tumor cells concentrate the cationic dyes to a far greater extent than do normal cells and retain it for days under non-equilibrium conditions[5–9]. The mechanism of selective uptake and retention by tumor cells may be related to the greater negative membrane potential of tumor cells at the mitochondrial membranes (−180 mV), responsible for binding the cationic Rh123, *versus* −60 mV for the mitochondrial membrane

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of normal cells[7]. The rhodamine 123 concentration in the incubation medium correlated directly with photoradiation-induced killing of cultured, malignant cells[10].

All the published reports to date demonstrate quantitation of Rh123 in whole cells by epifluorescence, either in a flow cytometer or in histological preparations, or in a spectrofluorometer[1,4,11].

The current report utilizes a reversed-phase separation method to quantitate Rh123 sequestered in cells in culture and in control or tumor tissue from animals.

EXPERIMENTAL

Preparation of stock solutions

Rhodamine 123 and Rhodamine 110 preparations were gifts from Eastman Kodak (Rochester, NY, U.S.A.). Standard solutions of Rh123 or Rh110 were made separately by dissolving 100 mg of stock compound in 10 ml dimethylsulfoxide and bringing the solution to 100 ml in a volumetric flask. The stock solutions were protected from light in brown glass bottles covered with metal foil to minimize photobleaching and stored at −20°C. Stock solutions were stable under these conditions for at least three months. Stock solutions were diluted to 10^{-4} or 10^{-5} with deionized water to make working solutions for constructing standard curves.

Chromatography

Analytical separations of standard and experimental solutions containing rhodamine compounds were conducted using a C_8 reversed-phase column at a flow-rate of 2 ml per min (DuPont Golden Series, 8 × 0.62 cm, 3 μm particle size, 60 Å pore size). A linear gradient of 0.05 *M* phosphate buffer, pH 2.85 (mobile phase A), and acetonitrile (mobile phase B), 10–80% B in 15 min, followed by 90% B for 5 min then 10% B for 8 min was used. A Waters Assoc. WISP was used for autoinjection of samples (Waters Assoc., Milford, MA, U.S.A.). A DuPont 880 system controller and pump in conjunction with a Shimadzu (Columbia, MD, U.S.A.) RF 530 spectrofluorometer set at 300 nm excitation and 500 nm emission (optimized with rhodamine standard in the flow cell) were used. A Dynamic Solutions (Pasadena, CA, U.S.A.) integration system was used to store data and integrate peaks.

For preparative chromatography, a 2.0-ml fixed loop injector was used to introduce the stock rhodamine solution (1 mg/ml, 2 ml sample volume) onto the head of a DuPont preparative C_8 column (25 cm × 242 nm, P.N. 880952-106, DuPont, Wilmington, DE, U.S.A.). A flow-rate of 8 ml/min with a linear gradient of 10–80% B in 75 min was used. Detection was performed using a Waters 440 UV monitor at 260 nm. Peaks eluting at 41.3 (Rh110) and 51.2 min (Rh123) were collected and pooled from 4 separations. Acetonitrile was evaporated under a stream of nitrogen. The two compounds were dissolved in a small volume of *n*-butanol whereupon the phosphate precipitated from solution. The butanol was evaporated under a nitrogen stream and the latter process repeated three times until the salt concentration in the sample was minimal.

UV–VIS and fluorescence spectrophotometry

Ultraviolet to visible scans of rhodamine compounds were carried out using a UV-240 Shimadzu spectrophotometer. Fluorescence scans were performed using the Perkin Elmer (Stanford, CT, U.S.A.) MPF-66 spectrofluorometer.

Mass spectrometry

Samples solvated in dimethylsulfoxide (DMSO) were examined by field desorption mass spectrometry using a double focusing Varian (Sunnyvale, CA, U.S.A.) MAT-731 mass spectrometer.

Cell culture

For *in vitro* studies of the retention of Rh123, two sublines of the Dunning R3327 rat prostate adenocarcinoma were used. Dunning R3327 MatLu and MatLyLu cells were cultured as described[12,13]. Cultures in stationary phase were treated with 10 μg/ml Rh123 and incubated for various times (5, 15, 30, 60, 120 min) then washed 3 times with culture medium without serum. Cells were scraped from the plates with two 1-ml volumes of *n*-butanol. Samples were brought to a volume of 5 ml in a volumetric flask with *n*-butanol. The samples were extracted with vigorous manual shaking for 5 min, in glass tubes with PTFE-lined caps. This extraction removed 98% of the rhodamine from the sample. Samples were sedimented at 3000 *g* for 10 min then supernatant fluids were collected and a portion used for analytical analysis of rhodamine compounds by high-performance liquid chromatography (HPLC).

Animal care and tumor innoculation

Adult, male athymic (nude) mice of the beige strain (bg/bg) were utilized in this study. Animals were housed in the Department of Surgery Athymic Mouse Facility. All National Institute of Health guidelines regarding care and handling of laboratory animals were followed. Dunning R3327-MATLu and MATLyLu rat prostate adenocarcinomas transplanted surgically, under anesthesia, from solid tumors grown in other mice, or cultured cells were injected subcutaneously in the inguinal fat. Transplanted tumors were allowed to grow to 0.3–1.0 mm diameter before mice were sacrificed by cervical dislocation. Tissue samples were collected and stored at −20°C until used.

Rhodamine treatment and tissue extraction

Mice bearing tumors received intraperitoneal injections of 10 mg/kg body weight RH123 dissolved in 50% DMSO. Mice were sacrificed by cervical dislocation, 24 h after injection. Tumors were removed and frozen. Tumor samples weighing approximately 100 mg were removed for extraction and treated as follows: (1) samples were homogenized for 30 s in 0.5 ml distilled water at 4°C with a Polytron homogenizer; (2) the homogenate was collected, then step 1 was repeated twice with 0.5 ml water each time; (3) the homogenizer probe was washed twice for 30 s each time in 800 ml water, once for 10 s with 95% ethanol then rinsed with a stream of water and wicked dry prior to preparation of another extract. Following homogenization, rhodamine was extracted from the homogenate in the following way: 5 ml of HPLC grade *n*-butanol were added to 1.5 ml of homogenate. Samples were vortexed for 1 min, then 5 ml of *n*-butanol were added, followed by 1 min of vortexing. The extraction proceeded overnight in the dark at 4°C in stoppered tubes. The suspension was vortexed, then sedimented at 2000 *g* for 5 min. The supernatant fluid comprised of butanol–water was removed and saved, protected from light. The pellet was rinsed with 1 ml of *n*-butanol, the sample vortexed, sedimented and the second supernatant fluid added to the first supernatant fluid.

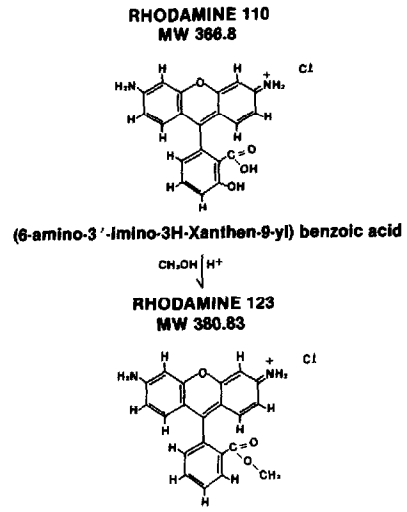

Fig. 1. Structures of rhodamines 123 and 110.

RESULTS

Fig. 1 depicts the structures of Rh123 [methyl O-(6-amino-3′-imino-3H-xanthen-9-yl) benzoate monochloride, molecular weight 380.83] and Rh110. Rh110 [6-amino-3′-imino-3H-xanthen-9-yl) benzoic acid, molecular weight 366.8] is the parent compound to Rh123. The positive charge of the 3′-imino group is responsible for the cationic nature of Rh123 at neutral pH. Rh110 has no charge at neutral pH.

Data in Fig. 2 indicate that detection of rhodamine is linear from 0.2 to 4.0 pmoles in the current system (correlation coefficient = 0.999).

Fig. 3a and b are spectral scans from 190 to 700 nm of stock solutions of Rh123 and Rh110. The stock Rh123 compound contains at least 6–8% of the parent

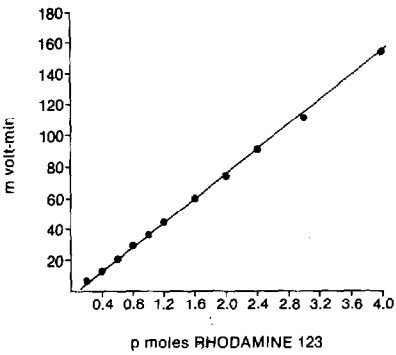

Fig. 2. A standard curve of rhodamine 123 was constructed by preparing standard rhodamine (100 mg) in 10 ml dimethylsulfoxide then diluting to 100 ml in a volumetric flask. Samples were diluted up to 10^{-5} in *n*-butanol then applied to the C_8 reversed-phase column and eluted with a linear gradient of acetonitrile (B), 10–80% B in 15 min, 2 ml/min, 25°C. Standard Rh123 eluted at 11.8 min whereas Rh110 eluted at 9.9 min.

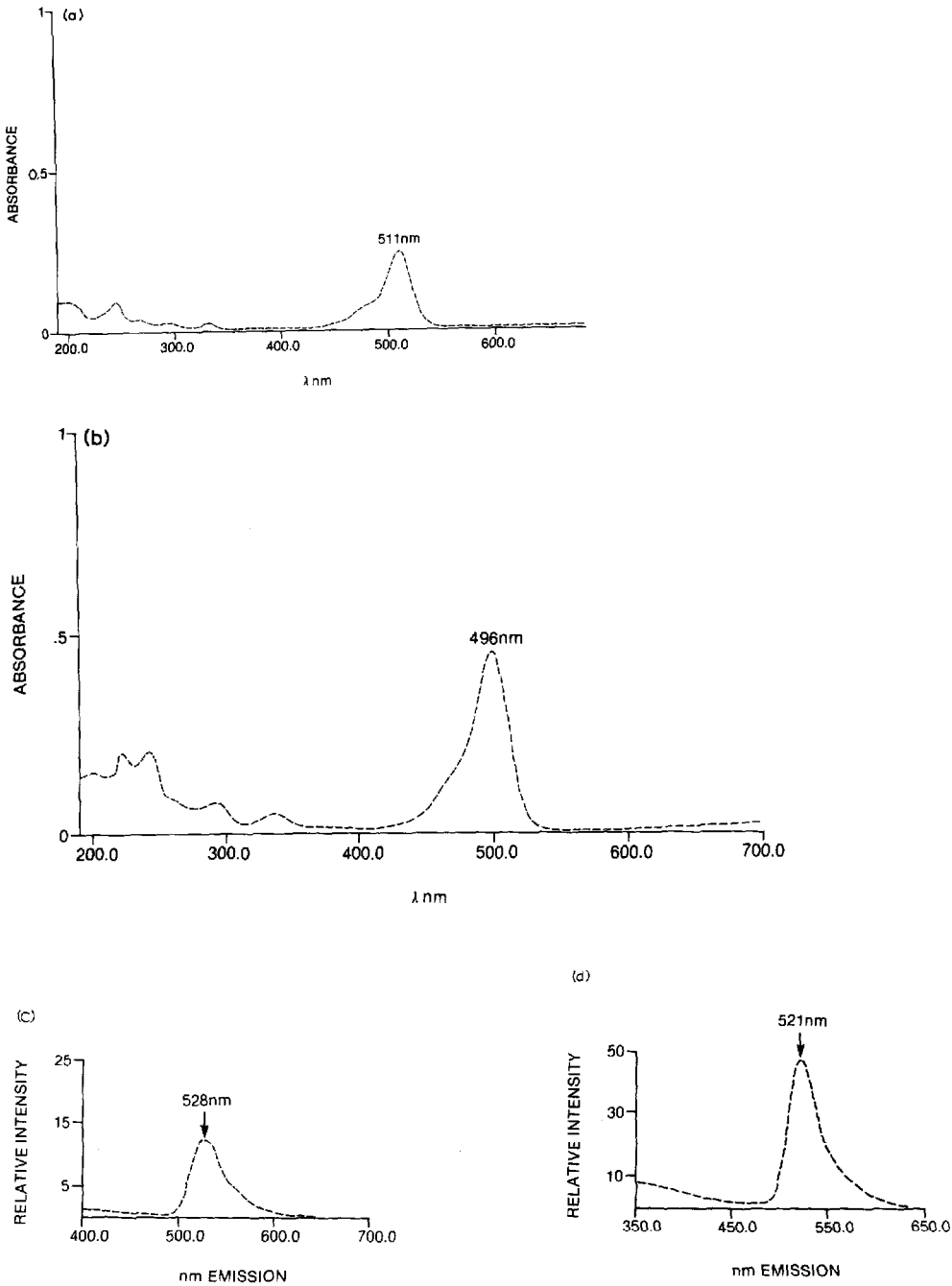

Fig. 3. (a) Spectral scan (UV–VIS), from 190 to 700 nm, of rhodamine 123 dissolved in ethanol. The absorbance peak was at 511 nm. (b) Spectral scan of Rhodamine 110 dissolved in ethanol. The absorbance peak was at 496 nm. (c) Fluorescence scan of rhodamine 123 in ethanol. Excitation was at 334 nm and the emission peak was at 528 nm. (d) Fluorescence scan of rhodamine 110 in ethanol. Excitation was at 294 nm and the emission peak was at 521 nm. Other excitation peaks were at 334 and 482 nm.

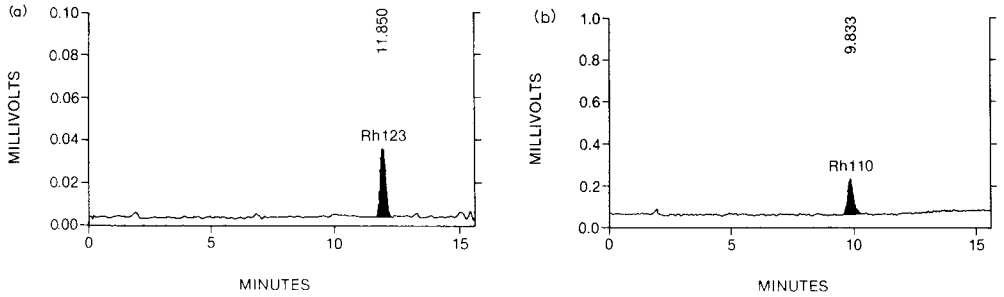

Fig. 4. (a) Chromatogram of a butanol extract of MLA prostatic cells grown in culture and incubated with 10 μg rhodamine 123/ml medium for 2 h at 37°C. Cells were then washed to remove unincorporated dye and extracted in *n*-butanol. A portion of the extract was subjected to chromatography to separate and quantitate the laser dye. A single peak of dye was detected. (b) Elution of stock rhodamine 110 under the same conditions as in (a).

compound, Rh110. The UV–VIS scan of Rh123 indicates a major peak at 511 nm. The scan of Rh110 indicates a major peak at 496 nm.

Fig. 3c and d are fluorescence scans of Rh123 and Rh110, respectively. Rh123 had an emission peak at 528 nm when excited at 334 nm (Fig. 3c). Rh110 had an emission peak at 521 nm when excited at 294 nm (Fig. 3d).

Fig. 4a indicates the peak of Rh123 eluting at 11.85 min in the linear gradient of phosphate–acetonitrile from a typical separation of 0.5 pmoles of standard. The contaminating Rh110 peak is not detectable at this level of sensitivity.

Fig. 4b is a separation of 2.5 picomoles of standard Rh110 which elutes at 9.83 min, approximately 2 min earlier than Rh123.

Fig. 5 is a chromatogram of a butanol extract of ML cells 24 h after a 2 h incubation with 10 μg Rh123/ml (5 ml medium per plate). Cell monolayers were washed three times with medium after the 2-h incubation to remove excess rhodamine. The peak eluting at 11.8 min is Rh123. The peak at 9.867 min coeluted with standard Rh110 and was the Rh110 metabolite of Rh123 that de-esterified.

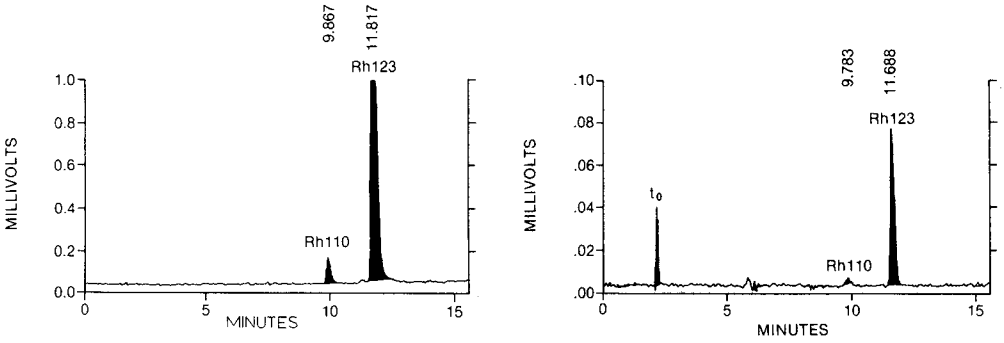

Fig. 5. Chromatogram in which both Rh110 and Rh123 appear. Prostatic cancer cells were treated *in vitro* with 10 μg Rh123/ml medium for 2 h. The resultant butanol extract contained both Rh110 and Rh123. Rh110 may have accrued from metabolism of Rh123 by de-esterification of the methyl group at the benzoate function of Rh123.

Fig. 6. Chromatogram of RH110 and Rh123 separated from a tumor comprised of human ML cells injected in the flank of a nude mouse.

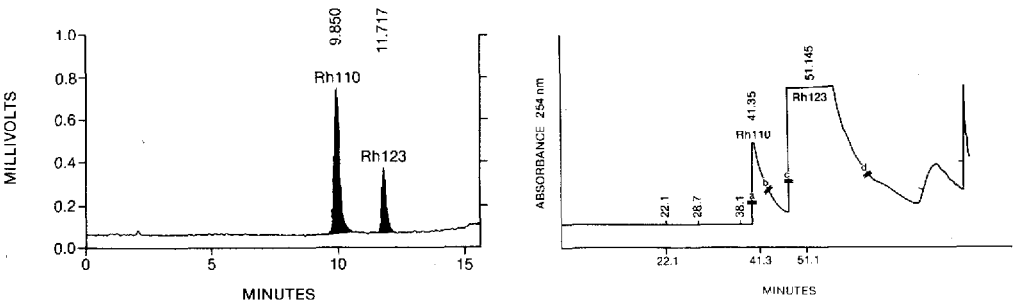

Fig. 7. Chromatogram with both Rh110 and Rh123 extracted from cells in culture incubated with Rh123. Standard Rh110 was added to the cell extract to verify the position of Rh110.

Fig. 8. Preparative separation of Rh110 and Rh123 after application of 2 mg of stock Rh123 to a C_8 DuPont preparative column and elution with 10–80% B in 75 min at 8 ml/min. Detection was at 254 nm. The major peaks of Rh123 and Rh110 were collected as shown by the double hash marks on the peak sides (R110 a–b; R123 c–d). Additional contaminating compounds were detected at 22.1, 28.7 and 38.1 min.

Fig. 6 is a chromatogram of a tissue extract of a solid tumor from the flank of a nude mouse previously injected with ML cells. The minor peak at 9.783 min was Rh110. The major peak at 11.688 min was Rh123. The material at 2.15 min was unretained.

Fig. 7 is a chromatogram of Rh123 and Rh110 from a cell extract with added standard Rh110, indicating coelution of standard Rh110 with the 9.85 min peak.

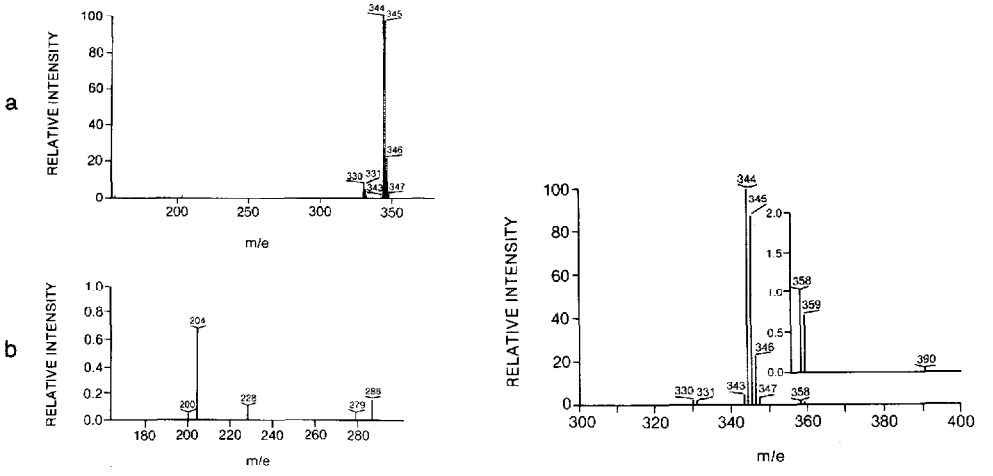

Fig. 9. Averaged field desorption mass spectrum for a commercial preparation of Rh123. The major ion peaks observed were m/e = 344 $(M)^+$ and m/e = 345 $[(M + 1)^+]$ (where m is mass and e is charge and M is the ion). (a) Shows an ion peak at m/e = 330, suggestive of Rh110, approximately 10% of m/e = 344 ion intensity; (b) shows other ions, each at less than 1% ion intensity of m/e = 344, at m/e = 28, 279 and 286.

Fig. 10. Field desorption mass spectrum for chromatographically purified Rh123. The major ion peaks observed were m/e = 344 (M^+) and m/e = 345 $[(M + 1)^+]$. Ion peak m/e = 330, suggestive of Rh110, is 1.8% of the m/e = 344 in intensity; m/e = 358, perhaps the ethyl ester, is less than m/e = 344 ion intensity.

Fig. 8 is a chromatogram showing the UV scan at 260 nm of the effluent from a DuPont C_8 preparative column loaded with 2 mg of Rh123. The primary peaks at 41.35 min and 51.145 min corresponded to Rh110 (a to b for further analysis) and Rh123 (c to d for further analysis), respectively. Other components were detected during the separation and were saved for later analysis. The presence of additional peaks, eluting at 22.1, 28.7 and 38.1 min, in the chromatogram of the preparative gradient elution confirmed the mass spectrographic data, indicating that contaminants other than Rh110 were present in the stock solution. The latter contaminants were detected as peaks when the absorbance scale was decreased.

Chromatographically pure Rh123 and Rh110 were subjected to mass spectrographic analysis to confirm structure. The data in Fig. 9a confirmed the presence of Rh123 [$M^+ = 344$ *m/e*; $(M + 1)^+ = 345$ *m/e*] as major ion peaks. This was the compound that eluted at 51.2 minutes in the preparative chromatogram (Fig. 8). Data in Fig. 9b and the inset of Fig. 10 indicated that ions of lower and higher mass respectively were also present as contaminants. The field desorption mass spectrographic data in Fig. 9a were consistent with the Rh123 structure (Fig. 9).

Data in Fig. 9a and 10 confirmed the presence of Rh110 as the ion peak at *m/e* = 330 in the Rh123 preparation. Rh110 was the compound that eluted at 41.35 min in the preparative chromatography (Fig. 8).

DISCUSSION

Rhodamine 123 is a laser dye that has a future as a therapeutic drug in the treatment of cancer[7]. The drug is toxic to tumor cells *in vitro* and to tumors *in vivo* as well. Moreover, dye treatment combined with application of laser light act synergistically to amplify the toxicity[10]. However, in order to be of use clinically, the constituents of the active material must be known. Commercially available Rh123 has been used principally as a mitochondrial stain and was not prepared commercially in highly purified form. Results of preparative chromatography indicated that at least 5 compounds were present in stock Rh123 at 1% or less concentration, including the parent compound Rh110 which represented approximately 6–8% of the total dye by weight (Figs. 9a and b, and 10). The phosphate–acetonitrile gradient elution system easily resolved the two principal rhodamines, with Rh110 eluting prior to Rh123 (Figs. 5, 7 and 8). The lesser contaminants were beyond the limits of detection in analytical separations but were readily detected in preparative separations (Fig. 8).

It has been stated that Rh110 is not biologically important because it does not enter the cell due to its lack of charge at neutral pH[7]. However, the compound may be formed by de-esterification of Rh123 inside the cell (Fig. 1). It appears that Rh110 is formed in cells incubated with Rh123 (Fig. 5). The effect of intracellular Rh110 on cellular metabolism is not known.

Preliminary experiments with Rh123 that contained 1.8% Rh110 indicated that cytotoxicity to cultured cells was reduced (data not shown). Higher concentrations of more pure Rh123 were also required to produce photoradiation-induced cell killing *in vitro*. These findings suggest the possibility that Rh110 may be a cytotoxic factor alone and/or in conjunction with Rh123.

Cells from human prostatic cancer grown *in vitro* can take up the dye. Studies

have shown that the dye is toxic to cells *in vitro* when they are exposed to blue-green light from an argon laser[14]. A solid tumor from the same cells, induced *in vivo* by inoculation of nude mice, will also incorporate the drug and necrose after administration of blue-green light. Therefore, toxicity in the *in vitro* culture system may be used as a predictor of efficacy *in vivo*. However, adequate tissue levels of Rh123 must be obtained to ensure tumor necrosis after light exposure. We are currently correlating the Rh123 concentration detected in cultured cells with that observed in solid tumors *in vivo*.

Future studies of this nature will employ pure Rh123 and cationic analogues that may be more selective and toxic for tumor cells than Rh123 alone.

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