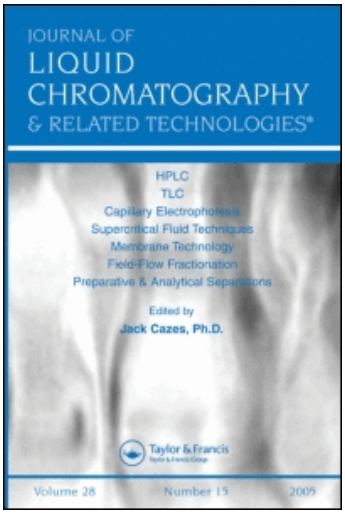

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T. W. Sweatman[a]; R. I. Larussa[a]; R. Seshadri[a]; M. Israel[a]
[a] Departments of Pharmacology and Medicinal Chemistry, The University of Tennessee-Memphis and The University of Tennessee, Tennessee

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AN ANALYTICAL SYSTEM FOR THE DETECTION AND QUANTITATION OF PHODAMINE-123 IN BIOLOGICAL SAMPLES

T. W. Sweatman, R. I. Larussa, R. Seshadri, and M. Israel

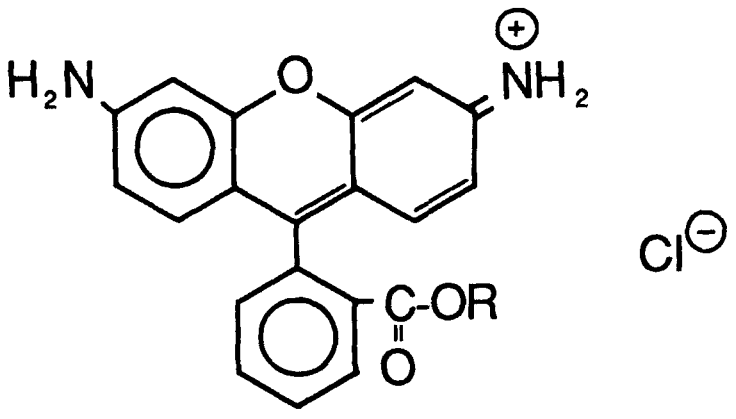
Departments of Pharmacology and Medicinal Chemistry
The University of Tennessee-Memphis
and
The University of Tennessee Cancer Center
Memphis, Tennessee 38163

ABSTRACT

Rhodamine-123, a mitochondrial-specific dye, is currently of interest as a potential anticarcinoma agent. We now report an analytical method suitable for monitoring this agent in a variety of biological matrices. The drug is easily extractable into organic solvent in high yield (>90%). Analysis is conducted by reversed-phase HPLC using a phenyl column and an organic/aqueous buffer mobile phase, with signal detection by flow fluorometry. The assay shows linearity over the concentration range of 5-4000 ng/ml, with a limit of sensitivity of 100 pg. Preliminary animal data confirms the suitability of the assay for detailed drug metabolism and dispositional studies.

INTRODUCTION

Rhodamine-123 (RH-123, Figure 1) is one of a group of xanthene-type compounds which are available commercially as laser



RH-123, R = CH_3

RH-110, R = H

Figure 1. Structures of rhodamine-123 and rhodamine-110.

dyes. Recently, several studies have reported RH-123 to have antitumor activity. Thus, RH-123 was shown to exhibit dose- and schedule-dependent activity against Ehrlich ascites and MB49 murine bladder carcinoma *in vivo* (1). In addition, RH-123 was found to produce a reduction in survival and colony-forming ability of murine epithelial bladder tumor cells *in vitro*, without having an effect upon counterpart 'normal' epithelial cells (2). The exact mechanism by which RH-123 may exert its cytotoxic effects remains unclear. However, the drug is known to be retained selectively by carcinoma cells *in vitro* (3), accumulating in mitochondria where it disrupts ATP synthesis (4-6). These

observations suggest that RH-123 may differ from most clinically-active anticancer agents which generally rely upon an interaction with a nuclear target for their cytotoxic properties. Accordingly, RH-123 may offer an alternative approach to the circumvention of drug resistance, a phenomenon which often has a significant impact upon disease prognosis. For this reason, further study, both of a mechanistic and pharmacologic nature, with RH-123 is clearly warranted. At present little is known of the pharmacology of RH-123 either in vitro or in animal models. As a prerequisite for such studies (7), we now report on the development of an efficient extraction procedure for RH-123 from biological matrices and a rapid sensitive HPLC assay for drug quantitation.

MATERIALS AND METHODS

Chemicals. RH-123 and crystal violet, used as internal standard, were obtained from Eastman Kodak, Rochester, NY. Both materials, as purchased, were found to be impure when analyzed by reversed-phase HPLC using the conditions described below. For purification, each compound was subjected to column chromatography on silicic acid, with chloroform-methanol elution, until it showed greater than 99.8% purity by HPLC analysis. AD 28 (N-trifluoroacetyladriamycin-14-octanoate), used as an internal standard in the tissue extraction studies, is a product of these laboratories (8). Siliconizing fluid (Sigmacote) and HPLC-grade acetonitrile were purchased from Sigma (St. Louis, MO) and

American Burdick and Jackson (Muskegon, MI), respectively. All other solvents and chemicals used in this work were of standard reagent grade quality.

Chromatography. The chromatographic equipment consisted of a Waters Associates (Milford, MA) dual M6000-A pump instrument equipped with microprocessor control and automated injection (Models 721 and 710B, and 730 WISP, respectively). This system was coupled to a flow fluorometric detector (Model FS-970, Kratos Schoeffel Instruments, Ramsey, NJ) set at an excitation wavelength of 482 nm (deuterium light source) and bearing a 550 nm emission filter. Separations were accomplished on a 10 cm x 8 mm i.d. phenyl-RADIALPAK column mounted in a Z-Module radial compression assembly (Waters Associates), with a mobile phase consisting of 0.05 M ammonium formate buffer, pH 4.0, and acetonitrile at a flow rate of 3.0 ml/min. Solvents were filtered (0.45 um) and degassed prior to use. To assure proper pump function, 100 ml of buffer was added to each 4-liter bottle of acetonitrile prior to filtration. Initial conditions were 75% buffer:25% acetonitrile; final conditions, 50%:50%, were achieved over 15 minutes using a linear gradient. For those experiments involving the use of AD 28 as an internal standard, a further increase in acetonitrile concentration (to 100% by 17 minutes) was used to rapidly elute this compound. A re-equilibration time of 4 min was used prior to automated injection of the next sample. The limit of detection of an injected standard of RH-123 was 100 pg (4 x background noise).

Extraction of RH-123 from Blank Human Plasma. RH-123 is sparingly soluble in completely aqueous media. For this reason drug was prepared in methanolic solution and appropriate dilutions were made in pH 7.4 Tris buffer. RH-123 (5-4000 ng) was added to triplicate 1 ml aliquots of blank human plasma (maintained at -70° and thawed immediately prior to use) contained in silicone-coated stoppered tubes. Following addition of drug, tubes were vortexed briefly and 1 ml of pH 7.4 Tris buffer (0.05 M) was then added. Samples were extracted with 2 x 7 ml of freshly prepared ethyl acetate:1-butanol (9:1, by volume). Organic phases for each sample were combined in silicone-coated test tubes and evaporated to dryness at 37° under a stream of dry nitrogen. Dried samples were capped with parafilm and stored frozen. Prior to analysis samples were reconstituted in 100 ul of methanol containing a known quantity of crystal violet as internal standard to provide a measure of the amount of sample injected by the automated system. Samples were transferred to WISP vials containing limited-volume inserts and analyzed as noted above.

Extraction of RH-123 from Blank Rat Plasma and Whole Blood. Fresh whole blood was obtained from anesthetized (Metofane inhalation anesthetic, Pitman-Moore, Washington Crossing, NJ) untreated Sprague-Dawley rats by cardiac puncture using a heparinized syringe and needle. Blood was transferred to heparin-treated vacutainer tubes and centrifuged at 3000 x g for 10 min to obtain plasma. To 1 ml aliquots (x 5) of either whole

blood or plasma, RH-123 was added in methanolic solution (25 ul) at a concentration of 25, 250 or 2500 ng. Samples were vortexed and plasma extracted as noted above for the human plasma samples. Whole blood was lysed by the addition of 2 ml of methanol and extracted with organic solvents as above. Samples were reconstituted with methanol (100 ul) containing crystal violet and analyzed by HPLC. Recovery was determined by peak area relative to that of directly injected RH-123 standards.

Extraction of RH-123 from Blank Rat Tissues. Untreated rats were sacrificed by cervical dislocation following exsanguination by cardiac puncture. Selected tissues (liver, kidneys, heart, lung, spleen) were removed rapidly and placed in ice-cold Tris buffer (0.05 M, pH 7.4). Tissues were blotted dry, weighed, and placed in 2 volumes of buffer. Homogenation was accomplished with a Polytron stainless steel homogenizer (Brinkman Intruments, Westbury, NJ) with ice-cooling of the samples. Complete cellular disruption was assured by subsequent sonication for 30 sec. The liver homogenate was divided into 5 x 3 ml aliquots (1 g of tissue) and RH-123 was added at 25, 250 or 2500 ng to each series of samples. Following vortexing, the samples were placed in the refrigerator ($+4^{o}$) for 30 min. For the remaining tissues, 1 ml of homogenate (0.33 g of tissue) was combined with 2 ml of Tris buffer prior to the addition of RH-123; kidney tissue was an exception, with 2.5 ml (0.83 g of tissue) being used. Each tissue homogenate was subdivided into 5 samples and 250 ng of RH-123 was added to each sample tube. All tissue samples were extracted with

ethyl acetate:1-butanol (9:1, by volume), as noted above. Prior to analysis samples were resuspended in 100 ul of methanol containing a known quantity of AD 28 as internal standard. Peak areas obtained for RH-123 were compared with those produced by injection of the appropriate concentration of pure drug standard.

Decay of RH-123 in Whole Blood Following IV Administration. We have noted previously (7) that intravenous administration of RH-123 (5 mg/kg) to rats results in higher drug levels in whole blood than in plasma over the entire study period (0-24 hr). In a preliminary study the procedures described above have been applied to the determination of RH-123 blood levels in samples withdrawn from treated animals (Metofane anesthesia) by cardiac puncture for up to 24 hr post-drug administration.

RESULTS AND DISCUSSION

The relationship between the concentration of RH-123 added to human plasma and the peak ratio of RH-123 relative to internal standard is shown in Figure 2. Samples were processed in triplicate for each drug level and are expressed as a mean value ± standard deviation; the latter was typically <4%. Data analyzed by linear regression (coefficient of variation >0.9999) showed no deviation over the concentration range studied.

The efficiency of the extraction procedure for RH-123 from rat whole blood, plasma, and tissue samples is shown in Table 1. Values are expressed as a percentage of the peak area of extracted

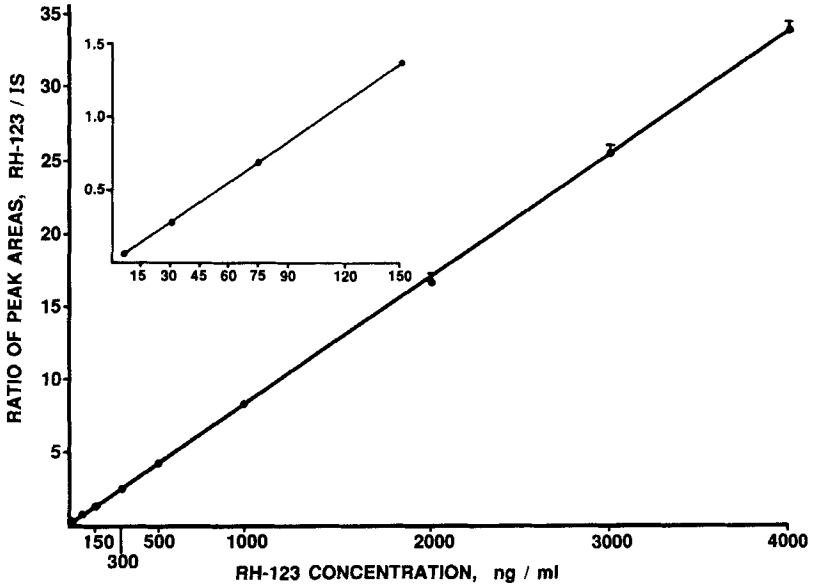


Figure 2. Standard curve for the extraction of RH-123 from blank human plasma (mean of 3 determinations). For details, see **Materials and Methods**.

TABLE 1

Percentage Recovery of RH-123 from Rat Whole Blood, Plasma, and Selected Tissues by Organic Extraction*

Sample	RH-123 Concentration Applied		
	25 ng	250 ng	2500 ng
Blood	92.4±9.4[#]	105.9±4.3	96.1±4.1[#]
Plasma	93.3±2.9	86.8±1.7	90.4±2.0
Liver	87.4±3.1	87.5±3.1	89.2±1.6
Kidney		106.8±5.4	
Heart		97.8±2.9	
Spleen		91.6±4.1	
Lung		87.1±8.4	

*Mean±S.D. for 5 (4[#]) separate determinations.

drug/directly injected standard, corrected for recovery of the internal standard. Over the 100-fold concentration range studied there was no discernable change in the efficiency of the extraction process which showed comparable values of ~90% for both the whole blood and plasma determinations. Standard deviation of the plasma samples (~2%) was lower than that for whole blood (~9%) at all three concentrations used. In common with plasma or whole blood, extraction of drug from liver homogenate was comparable at all three concentrations examined. Similar high extraction efficiencies were noted for the remaining tissues to which a single concentration of 250 ng of drug had been added.

The whole blood decay curve for RH-123 following intravenous administration of a single dose of 5 mg/kg to female Sprague-Dawley rats is shown in Figure 3. RH-123 was detectable at high levels (1245 ± 73 ng/ml) at 10 minutes, the earliest sample period. Drug levels appeared to undergo a biphasic decline, with an initial rapid decline in blood drug levels such that by 1.5 hr only 20% of the 10-minute levels were detectable. Thereafter, the decline was more gradual, with levels of RH-123 still detectable at 24 hr post-drug administration. In addition to parent drug (RH-123), two other fluorescent signals were detectable in the blood samples (Figure 4). At all sample times (0-24 hr), these other fluorescent signals represented no more than 5% of the total fluorescence present in the samples. These signals increased to reach a maximum between 1.5-2 hr post-drug administration and thereafter declined to undetectable levels. One of the unknown

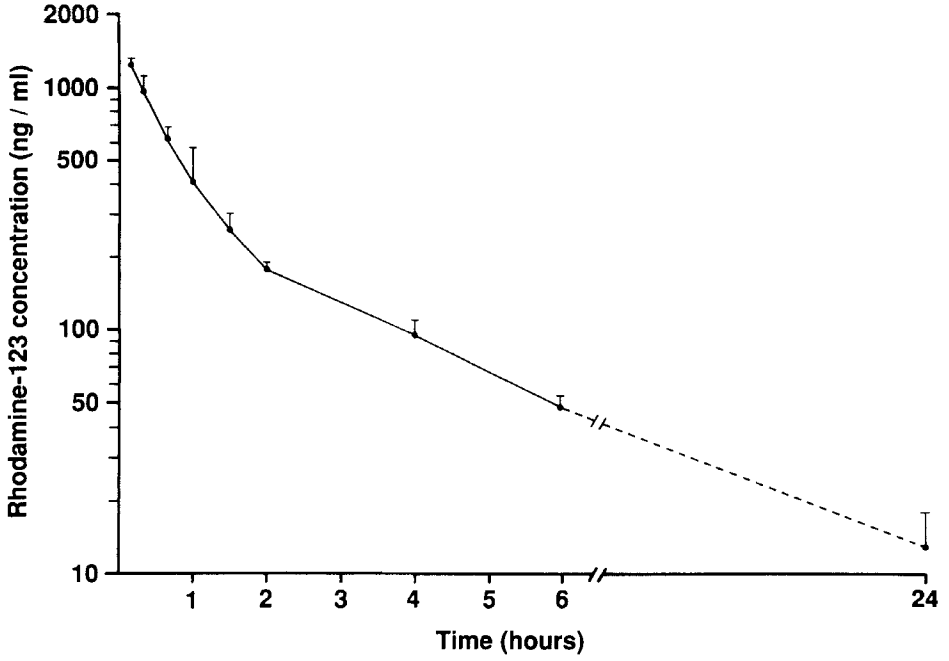


Figure 3. Mean concentration of RH-123 in the blood of rats following an intravenous bolus dose of 5 mg/kg (N=4). The dose administered was extrapolated from the optimal dose used in murine tumor therapy trials.

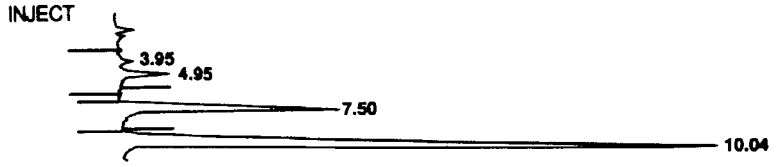


Figure 4. Reversed-phase separation of parent drug, metabolites, and internal standard extracted from a sample of whole blood obtained by cardiac puncture from a rat following administration of RH-123, 5 mg/kg iv. Retention times (min): 3.95, unknown; 4.95, RH-110; 7.50, crystal violet (internal standard); 10.04, RH-123.

metabolic products has tentatively been identified as Rhodamine-110, the de-esterified form of RH-123 (see structure, Figure 1), based on a comparison of spectral and chromatographic properties with an authentic standard. It should be emphasized that the in vivo data presented here are from preliminary studies done as part of our methods development program and therefore do not include sufficient data points for full pharmacokinetic evaluation; a more detailed report on the metabolism and disposition of RH-123 in the rat, using the methodology described here, will be presented elsewhere in the near future.

In conclusion, this report describes a new analytical methodology, by extraction and reversed-phase HPLC, for the analysis of RH-123 in biological fluids and tissues. The studies of extraction efficiency indicate that the developed method is highly efficient at extracting RH-123 from a variety of biological matrices, with a mean drug recovery of ~90% from whole blood, plasma, and tissue homogenates; similar results have also been obtained by us with urine, bile, and fecal samples. Comparable tissue extraction of drug over the 100-fold concentration range studied indictes that the linearity seen in human plasma samples also exists for extraction from other samples. Preliminary data from animal studies confirm the ability of the assay to separately quantify parent drug and organic solvent-extractable metabolites. This work also shows that the sensitivity of the analytical technique is appropriate for biological and pharmacological studies with RH-123. The limit of detection of a

directly-injected pure drug standard is 100 pg (4 x background). Thus, the limit of quantitation of a biological fluid or tissue extract containing RH-123 is less than 0.5 ng/ml; such a value represents less than 1/10th that observed in blood 24 hr after intravenous administration to rats of a therapeutic dose of RH-123, as extrapolated from the optimally active dose determined in murine antitumor trials.

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REFERENCES

1. Bernal, S. D., Lampidis, T. J., McIsaac, R. M., and Chen, L. B.: Anticancer activity in vivo of rhodamine 123, a mitochondrial-specific dye. Science, 222, 169 (1983).

2. Bernal, S. D., Lampidis, T. J., Summerhayes, I. C., and Chen, L. B.: Rhodamine 123 selectively reduces clonal growth of carcinoma cells in vitro. Science, 218, 1117 (1982).

3. Summerhayes, I. C., Lampidis, T. J., Bernal, S. D., Nadakavukaren, J. J., Nadakavukaren, K. K., Shepherd, E. L., and Chen, L. B.: Unusual retention of rhodamine 123 by mitochondria in muscle and carcinoma cells. Proc. Natl. Acad. Sci., 79, 5292 (1982).

4. Lampidis, T. J., Salet, C., Moreno, G., and Chen, L. B.: Effects of the mitochondrial probe rhodamine 123 and related analogues on the function and viability of pulsating myocardial cells in culture. Agents Actions, 14, 751 (1984).

5. Gear, A. R. L.: Rhodamine 6G. J. Biol. Chem., 249, 3628 (1974).

6. Higuti, T., Niimi, S., Saito, R., Nakasima, S., Ohe, T., Toni, I., and Yoshimura, T.: Rhodamine 6G, inhibitor of both H^+-ejections from mitochondria energized with ATP and with respiratory substrates. Biochem. Biophys. Acta, 593, 463 (1980).

7. Sweatman, T. W., Larussa, R., and Israel, M.: Systemic absorption of rhodamine 123 following instillation into rat urinary bladders. Abstracts, 14th Intl. Cancer Congress, Budapest, Hungary, 3, 944 (1986).

8. Israel, M., and Modest, E. J.: N-Trifluoroacetyladriamycin-14-alkanoates and therapeutic compositions containing same. U.S. Patent No. 4,035,566 (July 12, 1977).