Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXVII, No. 7

OPTIMIZED SYNTHESIS OF RADIOIODINATED RHODAMINE-123

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SUMMARY

Rhodamine-123 (Rh123) was iodinated with ^{123}I in presence of Iodogen. Yield of the labelled Rh123 varied greatly depending upon various reaction parameters. Optimum reaction conditions with respect to molarity and pH of the buffer, reaction time, amounts of iodogen, carrier iodide and Rh123, were determined through carefully controlled reactions. Radiochemical yields of 50-60% were obtained within less than 2 hrs. using 0.05M $NH_4H_2PO_4$ buffer at pH5, 200 µg iodogen, 250 µg Rh123 and no-carrier-added iodide. Separation of the labelled Rh123 in greater than 98% radiochemical purity was achieved by flash chromatography on silica gel. The overall isolated yield of radioiodinated Rh123 ranged from 25-35% with respect to the added radioiodide. TLC and HPLC methods were developed for analysis of the labelled products.

KEY WORDS: Rhodamine 123, Iodogen, Radioiodination, Quality Control **Iodine-123**

INTRODUCTION

Rhodamine-123 (Rh123, methyl-O-[6-amino-3-imino-3-H-xanthane-9-yl] benzoate monochloride, Fig 1) is a cationic fluorescent dye that accumulates in mitochondria of living cells, and is widely used in study of mitochondrial and cellular functions (1-6). The mitochondria of a variety of carcinoma cells retain Rh123 for prolonged periods, whereas, normal cells release it

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0362-4803/89/070789-14$07.00
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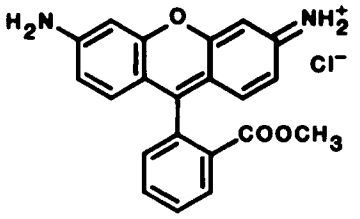
Received December 12, 1988

within 1-16 hours (7-10). Furthermore, it has been observed that Rh123 is relatively non-toxic to the healthy cells, but selectively kills certain carcinoma cells in vitro and in vivo (7-10). This selective accumulation of Rh123 in tumor cells and relative non-toxicity to the normal cells has generated interest in evaluation of radioiodinated Rh123 as a potential therapeutic and diagnostic agent (11-14).



Rhodamine 123 (RH 123)

Fig. 1 Structural formula of Rhodamine 123.

Radioiodination of Rh123 using iodogen, chloramine-T, and N-chloro-succinimide has been recently reported (11-14). Radiochemical yields ranged from 0 to 20% in relation to the added radioiodide. However, synthesis details were only sparcely reported. Therefore, we have investigated this reaction in greater detail to optimize the reaction parameters for preparation of radioiodinated Rh123 in reasonable yield, and in reproducible manner. We studied the effects of the type of iodinating agent, pH and molarity of buffer, reaction time, carrier-added iodide, and substrate concentration, on the yield of the radioiodinated Rh123. Chromatographic methods for analysis and purification of the radioiodinated Rh123 are presented.

EXPERIMENTAL

MATERIALS:

Rhodamine 123 (Rh123) was purchased from Eastman Kodak Company, Rochester, N.Y. U.S.A., and used without further purification. Iodogen and iodobeads were purchased from Pierce Chemical Company, Rockford, Illinois, USA.

Iodine-123 was produced in a cyclotron by $^{124}Te(p,2n)^{123}I$ reaction and obtained as [^{123}I]iodide in 0.25 - 0.35 N NaOH (16mCi/ml).

SYNTHESIS:

(a) IODOBEADS

To 200 µl of 0.01M $NH_4H_2PO_4$ buffer (pH range 2.8-9.3), two iodobeads were added, followed by addition of 50 µl of ^{123}I solution. After 5 min., 200µg of Rh123 in 200 µl of the same buffer was added and the reaction mixture was then sonicated. Aliquots were removed at 0.25, 2, and 24 hrs for assessment of the iodination by TLC.

(b) CHLORAMINE-T

In 1.0ml of 0.05M $NH_4H_2PO_4$, pH6, buffer 1.0 or 10.0mg of chloramine-T was added, followed by the addition of 50µl of radioiodide solution. After 5 min., 0.5mg of Rh123 in 500µl of the same buffer was added and the mixture was stirred at room temperature. Aliquots were taken at 1.0 and 2.0 hrs. to assess the progress of iodination.

(c) IODOGEN

Iodogen (200µg) in 100µl of methylene chloride was transferred into several tubes (1.3cm x 8cm) and solvent evaporated off under a slow stream of N_2. Tubes were agitated continuously to achieve a fine and even coating of iodogen on the inside wall of the reaction tubes. Ammonium dihydrogen phosphate buffer (500µl) was gently introduced into the iodogen coated tubes, Liquid Chromatography equipped with UV-50 variable wavelength detector connected in series with a flow-through NaI radioactivity detector (15). Radioactivity, injected vs eluted, was compared by assembling a loop by-passing the column. By injecting identical quantity of sample, injected vs eluted radioactivity could be compared directly. Analysis was performed on 5µ Alltech silica column (4.6 mm i.d. x 25 cm) eluted with 100% acetonitrile at a flow rate of 1.5ml/min. Under these conditions, unreacted iodide eluted at the solvent front, while iodinated Rh123 eluted at 2.82 min.

Radiochemical purification of iodinated Rh123 was readily achieved by flash chromatography. A glass column 1.0 cm i.d. x 10 cm, was packed with

silica gel equilibrated with methylene chloride, loaded with the reaction mixture, and eluted successively with 100% methylene chloride, 0.5%, 1.0%, and 2.0% methanol in methylene chloride, for optimum separation of radioiodinated Rh123 from radiochemical and chemical impurities. The column was eluted at a flow rate of 8-10 ml/min, and 10 ml fraction were collected. Most of the colored impurities and radioactive impurities with high Rf values eluted in 100% methylene chloride. The radioiodinated Rh123, begins to elute with 1% methanol in methylene chloride. Elution was enhanced by changing the eluent to 2% methanol in methylene chloride. Each tube was individually checked by TLC/autoradiography. Fractions containing mostly the labelled Rh123 were pooled and evaporated to dryness. Radiochemical purity of the purified iodinated Rh123 was assessed by TLC and HPLC.

RESULTS AND DISCUSSION

TLC analysis of the crude reaction mixture indicated several radioactive followed by the addition of $^{123}I^-$ solution. The mixture was shaken lightly a couple of times and allowed to stand for 5 min. Except in experiments involving the effect of Rh123 amount on yield of the iodinated product, 250µg of Rh123 in 500µl of the same buffer was then added to the reaction tube. The tubes were covered with aluminum foil and left in darkness. Aliquots were taken at 0.5, 1, 2, and 24 hrs for TLC and/or HPLC analysis of the reaction mixture to determine the extent of labelling.

The reaction mixture was filtered to remove loose particles of iodogen. The filtrate was extracted 2X with equal volume of CH_2Cl_2. Most of the unreacted iodide and Rh123 remained in the aqueous layer. The organic layer was dried over anhydrous Na_2SO_4 and then evaporated to dryness. The residue was dissolved in minimum volume of CH_2Cl_2 and subject to flash chromatography for purification.

CHROMATOGRAPHY

Thin layer chromatography was performed using Eastman Silica gel plates with fluorescence indicator. Usually 1 µl of the reaction mixture was

spotted. The plates were developed in either (a) 95% CH_2Cl_2: 5% CH_3OH or (b) 100% methyl ethyl ketone. In both systems, iodide remained at the origin. Rf value of the iodinated Rh123 in system a was 0.55-0.6 and in system b 0.67. Developed plates were autoradiographed on Polaroid 52 film. One cm sections were cut and radioactivity of all sections measured by NaI detector. Radioactivity in the spot corresponding to the labelled Rh123 was expressed as percentage of the total amount of radioactivity on the entire plate.

High performance liquid chromatography was performed using Varian 5000 species being formed. Unreacted iodide and iodinated Rh123 comprised major portion of the radioactivity. Radiochemical yield of the iodinated Rh123 was found to be dependent upon several reaction parameters. Various conditions investigated include molarity and pH of buffer, volume of I^-, amounts of Rh123, carrier iodide, and iodogen, and the reaction time.

All attempts to radioiodinate Rh123 using iodobeads (immobilized Chloramine-T on non-porous polystyrene beads, Ref.16) and free Chloramine-T (17,18) proved to be less satisfactory due to low radiochemical yield of the iodinated Rh123. These methods of iodination were abandoned as iodination catalyzed by iodogen (1,3,4, 6-tetracholoro-3α-6α-diphenylglycouril) proved to be more successful.

MOLARITY OF BUFFER:

Dependence of radioiodination of Rh123 upon molarity of the phosphate buffer is shown in Fig.2. Yield of the labelled product increased steadily, reaching a maximum at about 0.05 Molar concentration of buffer. At higher molarity of the buffer (>0.05M), the solubility of Rh123 diminishes substantially. Slight heat accompanied by sonication helps increase the solubility. However, we chose to use 0.05M buffer, as there was no particular gain in yield by having greater amount of Rh123 in the reaction medium. Moreover, having lesser amount of the substrate facilitates the final clean-up to obtain purified labelled Rh123.

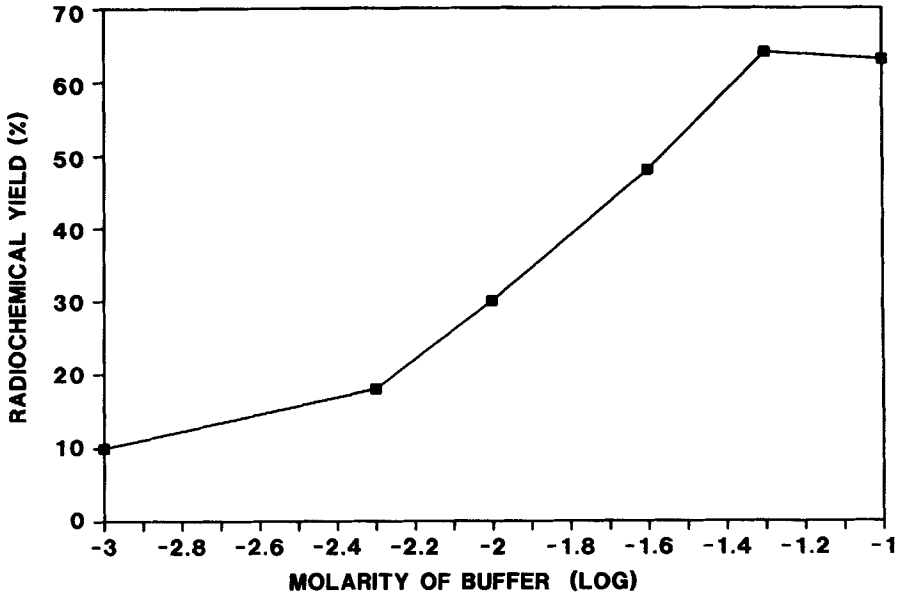


Fig. 2 Effect of the molarity of $NH_4H_2PO_4$, pH 5.0 buffer on radiochemical yield of Rh123

pH OF BUFFER

The effect of pH of buffer on yield of the radioiodinated Rh123 is shown in Fig. 3. Optimum pH seems to be in the range of 3-7. At pH above 8, Rh123 tended to precipitate out of the solution. For practical purposes, pH 5 buffer was normally used as the addition of the basic radioactive iodide solution tended to elevate pH of the reaction medium slightly. Also, larger volumes of radioactive iodide solution can be added without much affecting the yield.

VOLUME OF IODIDE:

Effect of the volume of iodide added to the reaction mixture on yield of labelled product depended upon molarity of the buffer. Obviously, the buffer with higher buffering capacity presented the least effect on the yield of the labelled product (Fig. 4). Up to 200µl of iodide was added in 0.05M buffer without much affecting the yield. This allowed us to prepare greater quantity of the labelled product.

AMOUNT OF SUBSTRATE:

Up to 250µg of Rh123 was added to the reaction medium. The yield of

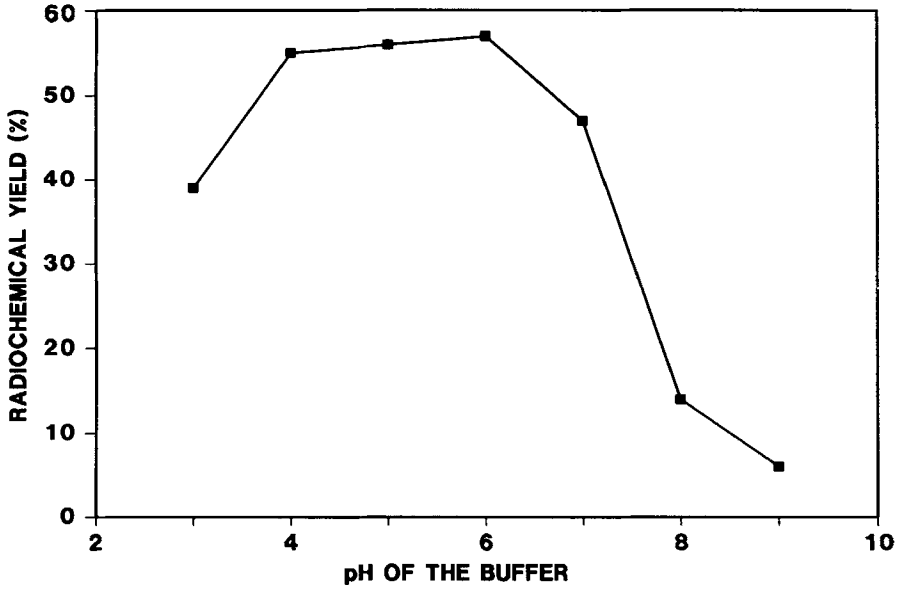


Fig. 3 Effect of the pH of 0.05M $NH_4H_2PO_4$ buffer on radiochemical yield

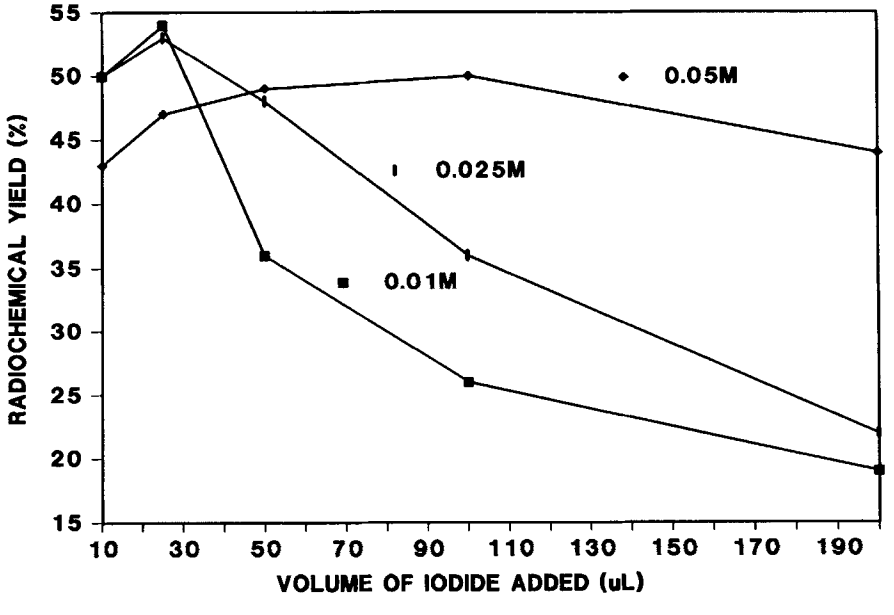


Fig. 4 Effect of the volume of ^{123}I solution in 0.25-0.35 N NaOH added to the reaction mixture

labelled product increased upon addition of the substrate as shown in Fig. 5. However, to keep the final clean-up simple and efficient, and the limited solubility of Rhl23, no more than 250μg of Rhl23 was added to the reaction mixture.

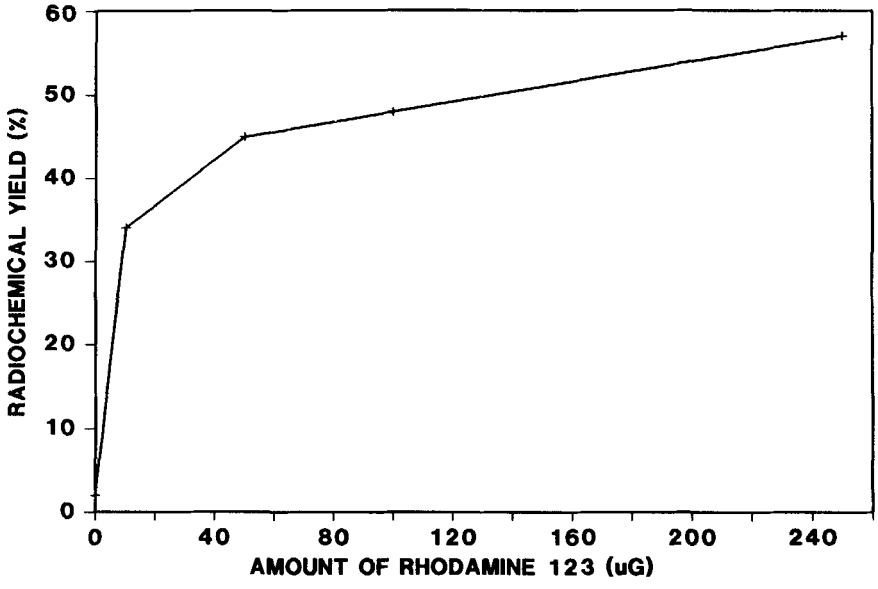


Fig. 5 Effect of the amount of Rhl23 on radiochemical yield

CARRIER-ADDED (I^-)

In quest for collecting sizeable quantities of the labelled Rhl23, and to study the effect of added carrier on yield, the reaction mixture was charged with various amounts of the added carrier (KI). As it can be seen in Fig. 6, yield of radioiodinated Rhl23 dropped drastically when one to one molar ratio of iodide to Rhl23 was used for the reaction. Furthermore, 1:1 molar ratio of I^- to Rhl23 resulted in formation of a red precipitate, also observed by Kinsey et.al. (12). An improvement in yield of the labelled Rhl23 was noticed up to a molar ratio of 10^{-2} of iodide to Rhl23. The ratio of iodide to iodogen is also in this range due to similar molecular weights of Rhl23 and iodogen. The initial increase in yield

upon addition of small quantities of carrier iodide may be explained by the shift of equilibrium. Decline in labelling yield upon addition of a large amount of carrier has been previously observed for other systems (17,18).

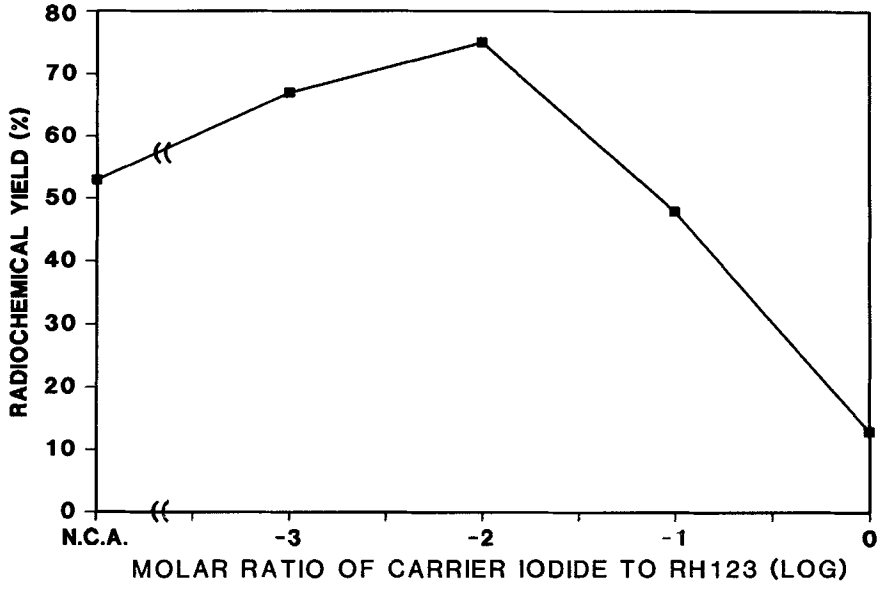


Fig. 6 Effect of the added carrier I^- on radiochemical yield X-axis. Log of the ratio of I^- to Rh 123

AMOUNT OF IODOGEN:

No radioiodinated Rh123 was possible in absence of iodogen as assessed by control experiments involving presence and absence of iodogen. This observation suggests that the labelled product is not just a hydro-iodide salt of Rh123. The yield of labelled product seemed to remain constant in the range of 50-400µg of coated iodogen (See Fig. 7). We routinely used 200µg of iodogen in all reactions.

REACTION TIME:

Initially, the yield of labelled Rh123 increased steadily up to approximately 2 hrs. However, no substantial gain was observed beyond two hours of reaction time (See Fig. 8).

Based upon these various reaction parameters and their affect on yield, the following conditions were derived for optimum labelling: To the tubes

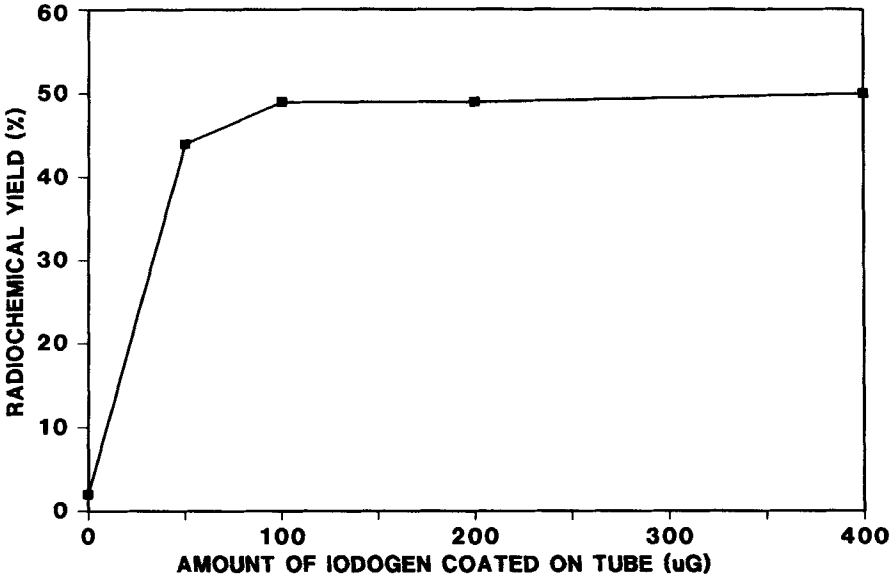


Fig. 7 Effect of the amount of iodogen on radiochemical yield

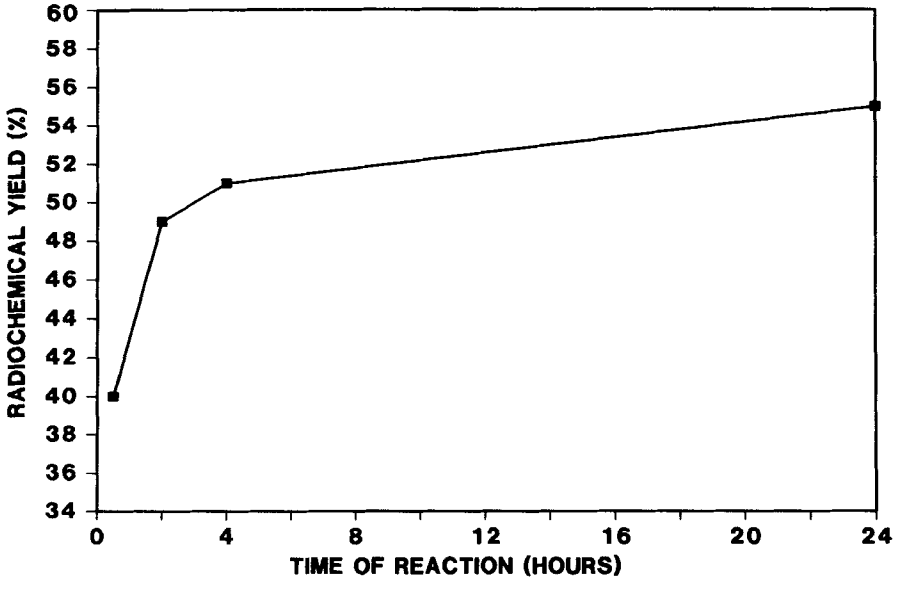


Fig. 8 Effect of the reaction time on radiochemical yield

coated with 200 µg of iodogen, 500µl of 0.05M $NH_4H_2PO_4$, PH 5.0 buffer was gently added, followed by the addition of 50-200 µl of radioactive iodide solution. After 5 minutes, 250 µg of Rh123 in 500µl of the same buffer was added, and the reaction was allowed to proceed for 2 hrs. The reaction mixture was filtered and extracted twice by methylene chloride. Radioiodinated Rh123 which is in the organic layer was subject to flash chromatography for chemical and radiochemical purification.

We noticed that in our hands, the work-up of iodination reaction involving precipitation ensued by addition of 0.5N NaOH (14) resulted in lower yield of the iodinated Rh123. Further investigated seems to indicate that the labelled Rh123 undergoes de-iodination in presence of base; up to 40% of the label was lost in work-up involving base. We had previously noted that the unlabelled Rh123 itself is unstable in base and decomposes partially to Rhodamine 110, the analog of Rh123, resulting from hydrolysis of the acetate ($-COOCH_3$) moiety to carboxylic moiety (TLC analysis on silica gel plates developed in 4:1 CH_2Cl_2:CH_3OH. Rf value of Rh123 = 0.4 and Rh110 = 0.1). Furthermore, we found that since in our system, we are working with only 0.25mg of Rh123, it is much easier to work-up the reaction through extraction step rather than the precipitation by base.

CHROMATOGRAPHY:

Yield of the labelled compound was assessed by TLC on silica gel plates, and by HPLC on silica column. The analytical yield data was used in obtaining profile of dependency of reaction parameters on yield of the labelled Rh123. Each reaction was analyzed using two blanks: (a) mixture of I^- and iodogen without Rh123, to ensure that the labelled product resulted only in presence of the substrate; and (b) mixture of I^- and Rh123 without iodogen to ensure that the labelled product was not an adduct of iodide and Rh123, and also that the labelled product occurred only when iodogen, Rh123, and iodide were all present simultaneously (See Fig. 9).

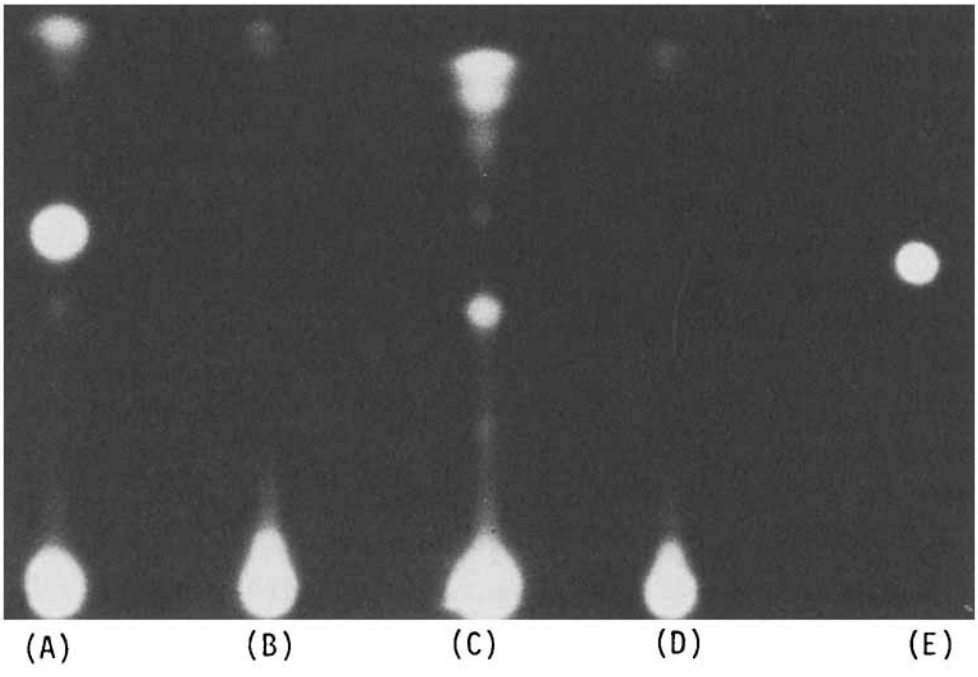


Fig. 9 TLC profile of the reaction mixture:
(A) Crude reaction mixture - I^-/Iodogen/Rhl23, 2 hrs;
(B) I^-/Rhl23, no iodogen;
(C) I^-/Iodogen, no Rhl23;
(D) I^-, no Rhl23 or iodogen;
(E) Radioiodinated Rhl23 purified by flash chromatography.

Purification was greatly simplified due to higher solubility of the labelled Rhl23 in methylene chloride in comparison to the unreacted iodide and Rhl23. These impurities remained mostly in the aqueous phase during extraction with methylene chloride. Flash chromatography further purified the labelled Rhl23 providing radiochemically as well as chemically pure radioiodinated Rhl23 (>98% radiochemical purity and no detectable quantity of unreacted Rhl23 or other fluorescent impurities). The overall radiochemical yield (25-35%) was lower due to loss in handling.

CONCLUSION

We have studied in detail the iodination of Rhodamine 123 catalyzed by iodogen. Yield of the radioiodinated Rhl23 depended upon careful control

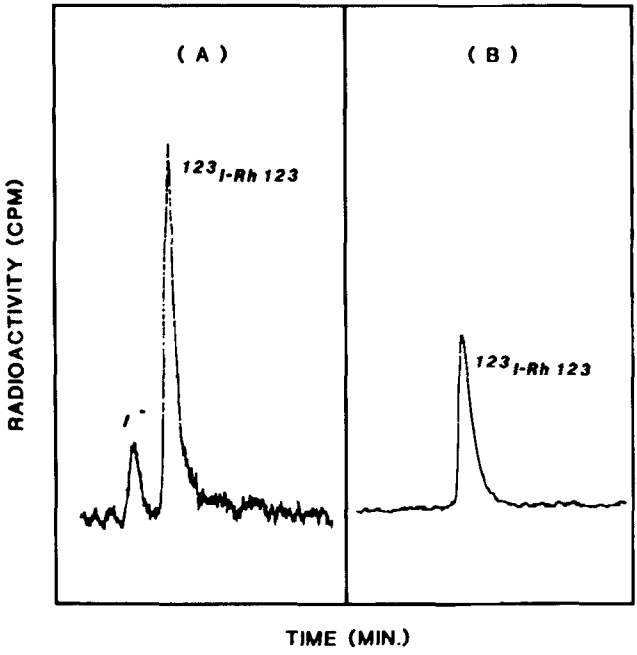


Fig. 10 HPLC profile:
(A) Methylene chloride extract before purification;
(B) Radioiodinated Rh123 purified by flash chromatography.
Alltech silica column, 5μ, 4.6mm i.d. x 25cm, eluted with 100% CH_3CN. Retention Times: I^-, 1.34 min; ^{123}I-Rh123, 2.82 min.

of several reaction parameters. Through these experiments we have derived optimized reaction conditions and chromatographic methods for routine no-carrier-added preparation of radioiodinated Rh123. Extraction in methylene chloride and flash chromatography allowed us to obtain >98% radiochemically and chemically pure radioiodinated Rh123 in the isolated yield of 25-35% in relation to the added iodide.

REFERENCES

1. Johnson, L.V., Walsh, M.L. and Chen, L.B. - Proc. Natl. Acad. Sci. U.S.A., 77: 990 (1980).

2. Johnson, L.V. Walsh, M.L., Bockus, B.J. and Chen, L.B. - J. Cell. Biol. 88: 526 (1981).

3. Goldstein, S. and Karczak, L.B. - J. Cell. Biol. 91: 392 (1981).

4. Collins, J.M. and Foster, K.A. - J. Cell. Biol., 96: 94 (1983).

5. Summerhayes, I.C., Lampidis, T.J. Bernal, S.D., Nadakavukaren, J.J. Nadakavukaren, K.K., Shephard, E.L. and Chen, L.B. - Proc. Natl. Acad. Sci. U.S.A., 79: 5292 (1982).

6. Nadakavukaren, K.K. Nadakavukaren, J.J. and Chen, L.B. - Cancer Res., 45: 6093 (1985).

7. Bernal, S.D. Lampidis, T.J., McIsaac, R.M. and Chen, L.B. - Science, 222: 169 (1983).

8. Lampidis, T.J., Bernal, S.D., Summherhayes, I.C. and Chen, L.B. - Cancer Res., 43: 716 (1983).

9. Lampidis, T.J. Bernal, S.D., Summerhayes, I.C. and Chen, L.B. - Ann. N.Y. Acad. Sci. 397: 299 (1982).

10. Bernal, S.D. Lampidis, T.J. Summerhayes, I.C. and Chen, L.B. - Science, 218: 1117 (1982).

11. Moonen P., Gorree, G.C.M. Hoekstra, A. - Nucl. Med. Comm. 8: 99 (1987).

12. Kinsey, B.M., Kassis, A.I., Fayad, F., Layne, W.W. and Adelstein, S.J. - J. Med. Chem., 30: 1757 (1987).

13. Padmanabhan, S. Chen, L.B., Corey, G., Garneau, J., Zabrowsky, L., Strauss, H.W. and Elmaleh, D.R. - J. Nucl. Med., 26: p124 (1985).

14. Thakur, M.L., Leeper, D.B., Rowley, R., and Park, C.H. - Nucl. Med. Biol. 15: 517 (1988).

15. Boothe, T.E., Finn, R.D. Vora, M.M., Emran, A.E., Kothari, P.J. and Wukovnig, S., in Synthesis and Applications of Isotopically Labelled Compouds, Proc. 2nd Int. Symp., Kansas City, MO, U.S.A., 3-6 Sept. 1985 453. (R.R. Muccino, Ed.)

17. Petzold, G. and Coenen, H.H., J. Label. Comp. Radiopharm., 18: 1319 (1981).

18. Youfeng, H., Coenen, H.H., Petzold, Z., and Stockling, J. Label. Comp. Radiopharm., 19 807 (1982).