Rapid Radioiodination of Rose Bengal at Room Temperature

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Published methods for radioiodination of rose bengal require reaction times of 1 hr or more at temperatures from 50 to 120°C. Through the use of an acidified ethanol solvent and potassium iodate oxidant, purified rose bengal is radioiodinated at room temperature within 15 min with chemical yields ranging between 93 and 97%. Radiochemical impurities are sufficiently minimized to permit preparation in a single 10-ml serum vial, requiring no additional purification steps. The method reported here is readily adaptable to a cold-kit preparation.

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I-131 rose bengal has been a valuable diagnostic agent for hepatobiliary studies (*1,2*), but its use has declined over the past few years due to concern over the absorbed radiation dose from I-131 and to the inefficient detection of the 364-KeV I-131 photon with the gamma camera. Rose bengal labeled with I-123, however, does not have these drawbacks and has been evaluated in a limited clinical study (*3*), which concluded that the larger photon flux from larger permissible amounts of the I-123 agent would prove advantageous in the differentiation between hepatocellular and extrahepatic obstructive jaundice.

The 13-hr half-life of I-123 must of course be considered a disadvantage, unless a means can be devised to reduce substantially the period from end of production to clinical administration. Matson (*4*) has pointed out the need to develop a series of cold kits to overcome the factor of time before widespread use of I-123 can become economically practical. We have addressed the general problem of radioiodination chemistries that, we hoped, would be adaptable to kit procedures.

In a program designed to study the chemical mechanisms involved in labeling biologically active compounds with radionuclides, we discovered that rose bengal (tetrachlorotetraiodofluorescein) can be radioiodinated rapidly in an acidified ethanol solution at room temperature (20°C). Previously reported methods of preparing radioiodinated rose bengal involve temperatures from 50 to 120°C and reaction times of 1 hr or more (*5–7*). The new simplified kit method of preparation described here, coupled with the availability of high-concentration, reductant-free $Na^{123}I$ available from LAMPF, BLIP, and other accelerators (*8*) would facilitate the application of the rose bengal hepatobiliary diagnostic test.

METHODS

Due to the observed presence of impurities in the commercially procured material, the rose bengal was purified by precipitation from a 10-mg/ml solution in ethanol by the addition of an equal volume of 1 *M* hydrochloric acid followed by centrifugation, removal of the supernate, and a water wash. Ten milligrams of the precipitated rose bengal was dissolved in 2 ml of water previously adjusted to pH 8 with sodium hydroxide, and 0.25 ml applied to a 1.5- × 35-cm column of molecular sieve*. Using the pH 8 water as the eluant, the rose bengal was eluted from the column in 25 ml of the second column volume. Samples taken at the beginning, middle and end of the fraction were adjusted to pH 8 with phosphate buffer and analyzed by visible absorption spec-

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troscopy in the 480- to 600-nm range using a recording spectrophotometer. All three samples had an absorbance peak between 548–550 nm corresponding to tetrachlorotetraiodofluorescein (*9*). An aliquot of the supernate from the rose bengal precipitation was air dried, dissolved in phosphate buffer, pH 8, and analyzed by spectrophotometry. The absorbance peak was 542 nm, probably corresponding to a compound bearing fewer than four iodine and four chlorine atoms (*9*).

Ten milligrams of purified rose bengal dissolved in 2 ml nondenatured ethanol, along with 0.2 ml of 1.5-mg/ml potassium iodate and 0.06 ml 1 *M* hydrochloric acid, were placed in a 10-ml serum vial, which was then stoppered and crimp-sealed. Using a 1-ml syringe and needle, 200 to 600 μCi of reductant-free [^{125}I] sodium iodide was added in a maximum volume of 0.3 ml. Samples of 0.2 ml were removed from the vial at 30 sec, 5, 10, 15, 30, and 60 min following the addition of the radioactivity and placed in a micro centrifuge tube. The rose bengal was precipitated in each sample by the addition of 0.2 ml 1 *M* hydrochloric acid, centrifuged, and the supernatant placed in a similar centrifuge tube. Each precipitate was resuspended in 0.2 ml water, centrifuged, and the wash added to the first supernate. The rose bengal precipitate was dissolved in 0.5 ml phosphate buffer, pH 7.3. Each set of product and supernate plus wash was assayed for radioactivity to determine the chemical yield of product. To determine the total labeling yield, reaction chromatograms were performed on the 10- and 15-min samples before precipitation, using ascending paper-strip chromatography in an aqueous solution of 3% ammonium hydroxide plus 25% ethanol. The R_f values of rose bengal, iodine and iodide were found to be 0.64, 0.96, and 0.96, respectively. Iodine is apparently converted to iodide in this chromatographic solvent, so iodine and iodide have the same R_f value. This point was confirmed by preparing duplicate reaction samples containing no rose bengal and performing the chromatography both in 3% ammonium hydroxide plus 25% ethanol and in 85% methanol. One radioactive peak was found in the rose bengal solvent at R_f 0.96 and two peaks were observed in the methanol solvent with R_f values corresponding to iodine and iodide. R_f values in 85% methanol were previously determined in this laboratory to be $\sim$ 0 and 0.82 for iodine and iodide respectively. The reaction procedures described above were performed in triplicate at 20°C, 50°C, and 92°C to study the effect of temperature. In addition, a triplicate set of experiments was performed as described at 20°C using nonprepurified rose bengal.

Biologic confirmation of the movement of the labeled product through the hepatobiliary system was obtained in a *Rhesus* monkey. The labeled product was prepared in a Wheaton-Hopkins tagging vial to facilitate aseptic preparation during the precipitation and water-washing steps, in addition to showing that the concurrent removal of ethanol and free radioiodide could be achieved in a single vial. The results of the product thus prepared were compared with results in a similar test on the same monkey using I-131 rose bengal†. In each case, the monkey was injected with a solution containing approximately 270 μCi of I-131-labeled product and less than 1 mg rose bengal. The monkey was imaged at 20, 80, and 100 min postinjection with a rectilinear scanner equipped with an I-131 collimator.

The labeling procedure up to this point was accomplished by using rose bengal in an ethanolic solution. However, since it had been demonstrated earlier in this laboratory that a true solution was not necessary to achieve high labeling yields, a wafer tablet containing purified rose bengal and KIO_3 was formulated to test the feasibility of a simplified and more rapid method of preparation with the subsequent reduction of the amount of ethanol required. Using a stainless steel tablet mold 0.04 cm thick, 18–20 tablets each weighing from 8.8 to 11.0 mg (9.4 mg, average) were manufactured by spatulating 250 mg of purified rose bengal powder with 0.25 ml of KIO_3 (75 mg/ml). Upon reaching a smooth paste with the desired consistency, the mass was molded.

A dried wafer was placed in a 10-ml rubber-stoppered serum vial together with a Teflon-coated magnetic stirring bar. Six hundred μl of acid-alcohol

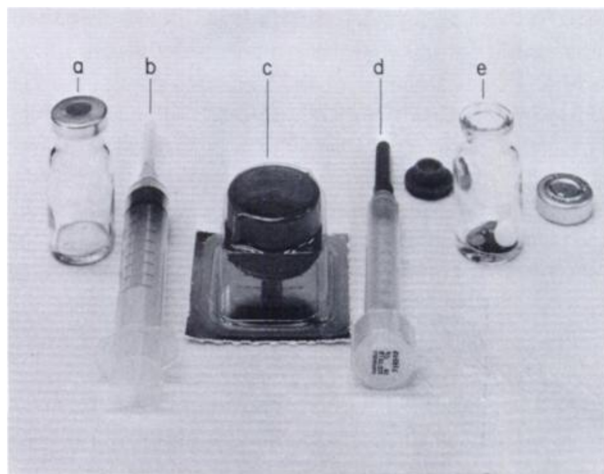

FIG. 1. Proposed rose bengal kit preparation showing, from left to right: (a) presterilized, stoppered, and crimped 10-ml serum vial; (b) 10-ml syringe containing sterile isotonic phosphate buffer solution; (c) sterilized Millipore filter; (d) 1-ml syringe containing acid-alcohol solution; and (e) 10-ml serum vial containing rose bengal-KIO_3 wafer and Teflon magnetic stirring bar.

solution (1.0 *M* HCl and ethanol, 1:11 v/v) were added through the septum and the mixture stirred until a creamy suspension was produced; this was followed by the addition of 200–600 μCi of reductant-free $^{125}I^-$. On an atom-per-atom basis, 600 μCi of carrier-free $^{125}I^-$ used in this study contains approximately 7 times the number of iodine atoms that would be contained in 10 mCi of carrier-free $^{123}I^-$. The mixture was allowed to stand for 15 min at room temperature. A solution of 4.2 ml of sterile isotonic phosphate buffer (0.15 *M*) plus 0.06 ml of 1 *M* NaOH was then added, giving a final product at pH 7.5. The product can be aseptically prepared with terminal sterilization by Millipore® filtration. (Figure 1 shows the equipment needed for kit preparation.) Aliquots of the final products were chromatographed, passed through the molecular sieve, and examined by visible spectroscopy, as described earlier.

RESULTS

The impurity found in the rose bengal starting material was observed to reduce the overall labeling yield of the rose bengal product. After 15 min of reaction time at room temperature, the chemical yield of the labeled rose bengal averaged only 80.5%; whereas, using the purified starting material, the rose bengal yields following precipitation and washing, averaged 91.1% after 15 min in both the 20°C and the 50°C tests, and showed no further increase with time. Figure 2 shows the product yields as a function of time. Curve A is the average yield of three determinations at 20°C and three determinations at 50°C using prepurified rose bengal. Curve B shows the average yield of three determinations at 20°C using nonpurified rose bengal. The experimental data are presented in Table 1. Results of the 92°C studies are not shown for reasons explained below.

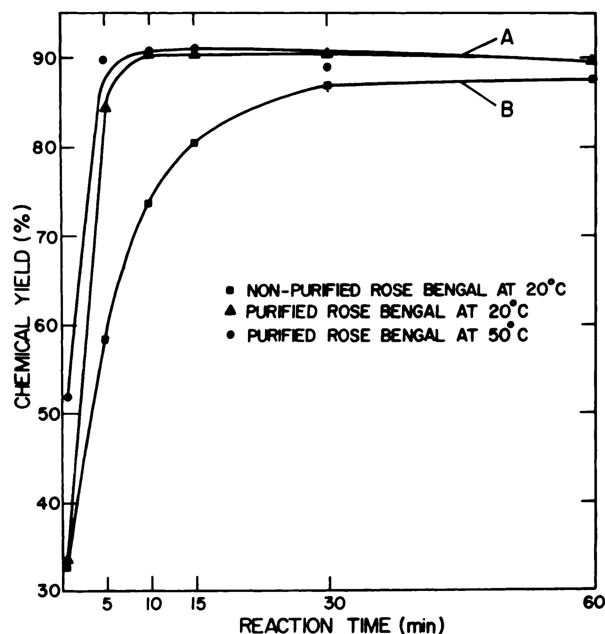

FIG. 2. Chemical yield of [^{125}I] rose bengal as a function of time after precipitation and dissolution in buffer.

Reaction chromatograms, indicating the labeling reaction yield before precipitation and wash, showed less than 5% free iodine and iodide for all 20° and 50°C samples taken at 15 min after addition of the radioactivity. Reaction chromatograms of the labeling performed at 92°C showed an additional colored spot, R_f 0.75, which contained 10% of the radioactivity after 15 min and ~ 50% of the activity after 60 min. The chromatograms also demonstrated more than 10% free iodide. Aliquots of products from the boiling-water reaction (92°C) were separated by molecular filtration and examined for absorbance peaks as described earlier. Three fractions having absorbance peaks at 532, 542, and 545 nm were separated, and none corresponded to the 548–550 nm absorbance peak of tetrachlorotetraiodofluorescein. Despite the fact that the peak activity on the chromatographic strip occurred at an R_f value corresponding to that of rose bengal, we are convinced by the visible spectroscopy data that rose bengal was no longer present. Thus, since the labeled product at 92°C was not rose bengal, the product yield as a function of time could not be presented.

Molecular filtration of aliquots of the final products prepared at 20° and 50°C both indicated one fraction with an absorbance peak at 550 nm.

Scans obtained from the primate study are shown in Fig. 3: A and B are scans of the commercial I-131 rose bengal at 20 and 100 min postinjection, respectively, and C and D are the scans of the rose bengal I-131, prepared using the Wheaton-Hopkins tagging vial, at the same postinjection periods. Scans obtained from both products show a rapid accumulation by the liver followed by a decrease in liver activity and the appearance of the radioactivity in the small intestine. The area of concentrated activity in C corresponds anatomically to accumulation in the gallbladder. In general, the two products behaved similarly.

Losses in processing time and overall chemical yield associated with rose bengal precipitation, washing, and re-suspension encountered in the Wheaton-Hopkins vial method prompted a study of a procedure employing a wafer tablet of purified rose bengal and KIO_3. Twelve separate experiments were performed at room temperature for 15 min, and chromatograms of the final products showed only 3–7% free $^{125}I^-$, which is within the limit of 10% estab-

TABLE 1. CHEMICAL YIELDS OF LABELED ROSE BENGAL AT 20 AND 50°C

	Yield percentage at indicated reaction time (min)					
	0.5	5	10	15	30	60
Prepurified rose bengal, 20°C	33.3	82.6	87.5	90.9	87.5	90.6
	54.2*	86.9	91.3	91.6	87.5	87.5
	63.6†	83.3	91.7	91.7	91.3	91.3
Average	—	84.3	90.2	91.4	88.8	89.8
Prepurified rose bengal, 50°C	51.7	86.8	89.8	89.8	88.0	86.6
	73.9*	91.3	91.3	91.6	91.3	90.8
	91.3†	91.6	91.3	91.6	91.3	90.8
Average	—	89.9	90.8	90.9	90.0	89.4
Nonprepurified rose bengal, 20°C	36.2	60.0	77.5	84.3	88.6	86.1
	31.5	61.8	76.9	81.0	85.0	91.1
	29.8	53.9	66.6	76.2	87.3	85.5
Average	32.5	58.5	73.6	80.5	86.9	87.5

* 1-min reaction time.
† 1.5-min reaction time.

lished by the United States Pharmacopia (*10*). Molecular filtration and absorbance studies showed the products from all these experiments to be of one fraction having absorbance peaks in the 548–550 nm range, which has been shown to correspond to that of pure rose bengal (*9*).

Although extended stability studies of the rose bengal and KIO_3 tablets have not been performed, our research has shown them to be chemically stable for a period of at least 21 days. No degradation of the I-125-labeled rose bengal was observed in a 72-hr period, the most probable time period within which one would expect an I-123-labeled product to be used.

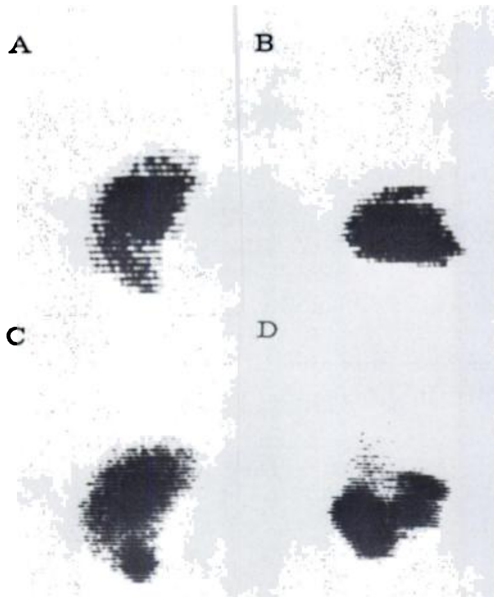

FIG. 3. Liver scans of a *Rhesus* monkey: 20 min (A) and 100 min (B) after the injection of Squibb rose bengal I-131; and 20 min (C) and 100 min (D) after injection of rose bengal I-131 prepared at LASL in a Wheaton-Hopkins tagging vial.

It is essential that the radioactive iodide solution used in the procedure reported here be free of reducing agents, otherwise the role of the KIO_3 will be inhibited and diminished yields of labeled rose bengal will result.

CONCLUSION

Rose bengal degradation products, possibly resulting from some dehalogenation process, may be present in commercially available material, and a purification step (precipitation of rose bengal from ethanol) is recommended to obtain maximum labeling yields. The impurities are more soluble in ethanol and can be readily separated under the described conditions.

The experimental evidence presented here demonstrates that rose bengal is radioiodinated rapidly at 20°C (room temperature) in an acidified ethanol solution. The reaction is complete in 15 min with reaction yields of better than 95% and product yields of better than 90%, following the removal of the ethanol and redissolution of the product in phosphate buffer (using the Wheat-Hopkins tagging-vial preparation). The molded tablet method, on the other hand, eliminates the precipitation and water washing steps by reducing the ethanol content to an acceptable minimum. In addition, the chemical yield of labeled rose bengal consistently ranges between 93 and 97%. There is no advantage in performing the reaction at 50°C, and an apparent chemical breakdown of the rose bengal occurs when performed in a boiling-water bath (92°C).

With this new procedure, which is adaptable to a cold-kit preparation, the labeling of the rose bengal can be performed in the clinical unit by a trained technologist, using reductant- and carrier-free, high-concentration $Na^{123}I$. Thus greater economy can be realized, since decay losses between end of bombardment and clinical administration of the iodine-123 would be substantially reduced.

FOOTNOTE

* Bio-Gel P-2®.
† Robengotope, E. R. Squibb, Princeton, N.J.

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