A SIMPLE CHEMICAL METHOD OF LABELING HEMATOPORPHYRIN DERIVATIVE WITH TECHNETIUM-99m

Dennis W. Wong

Division of Nuclear Medicine, Department of Radiology, Charles R. Drew Postgraduate Medical School, University of California at Los Angeles, 12021 S. Wilmington Avenue, Los Angeles, Calif. 90059

SUMMARY

Hematoporphyrin derivative(HPD), a fluorescent compound known to be selectively accumulated in neoplasms, is labeled with ^{99m}Tc by a chemical method. Radiochemical analyses indicated that the labeled product contains an average of 91% ^{99m}Tc-HPD, 6% unbound ^{99m}Tc-(Sn)complex species and less than 3% free ^{99m}Tc. Data from acid precipitation analysis demonstrate the presence of two radiolabeled HPD fractions; an insoluble black precipitate and an acid soluble fraction with an average of 66% and 25% respectively. The labeling mechanism is not known. Presumably, labeling of HPD involves the formation of a coordinated complex with reduced ^{99m}Tc forming a radioactive metalloporphyrin compound. ^{99m}Tc-HPD, like the parent compound, exhibits strong fluorescence when exposed to an appropriate ultra violet light source.

Key Words: ^{99m}Tc-Hematoporphyrin derivative, tumor imaging agent.

INTRODUCTION

The preferential affinity of porphyrins and hematoporphyrin for neoplastic tissue has been known for more than four decades.[1-4] When injected intravenously into tumor-bearing animal, a brilliant red-orange fluorescence is produced by ultra violet light activation of the porphyrin compound accumulated in the tumors. Hematoporphyrin derivative (HPD) appears to be a better tumor localizing agent

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than any porphyrin compounds investigated.[5-10] Despite the initial optimism over possible clinical applications of HPD in detecting tumors, the usefulness of the unlabeled compound is limited. The use of HPD-fluorescence technique involves invasive procedures. The fluorescence emitted by HPD must be activated in situ by a strong UV light source which requires highly sophisticated endoscopic fiberoptic equipments. Endoscopic procedures often produce tissue damages which lead to hemmorhage and subsequent masking of the tumor. Quenching of the fluorescence by normal tissue, body fluids and blood is a major obstacle in achieving significant reliablity and reproducibility of this technique. Another major problem is the inability to document photographically the fluorescence observed endoscopically. None the less, the HPD-fluorescence technique has proved valuable in initial clinical trials. HPD labeled with a suitable radionuclide such as ^{99m}Tc may resolve these problems and offer a simpler and practical means of tumor detection.

METHOD AND MATERIALS

HPD was prepared according to the method of Lipson.[5] One gram of hematoporphyrin dihydrochloride* was treated with 14 ml of a 19:1 mixture of glacial acetic acid and concentrated sulfuric acid for 15 minutes. HPD was precipitated from the acidic solution by the addition of 300 ml of a 3% sodium acetate solution, filtered, thoroughly washed with distilled water and dried at room temperature overnight in the dark. The yield averaged 80% HPD. One hundred milligram of HPD crystals was dissolved in 9 ml normal saline(0.9% NaCl) and alkalized to pH 11.5 with 1 N NaOH.After complete dissolution, the HPD solution was quickly lowered to pH 7.4 with 1 N HCl. Additional normal saline was added to bring the volume to 10 ml. Occasionally, minute particles were formed as a result of rapid pH adjustment or when the pH dropped below 7. The neutralized HPD solution was sterilized with 0.22 um Millipore® filter into a sterile evacuated serum vial and stored at room temperature in the dark. Ultrafiltration also removed any microcolloids presented in the neutralized HPD solution. After one month, the pH of the HPD solution decreased

* Calbiochem-Behring Corp. Calif.

to 6.8 causing formation of microcolloids but could be resolubilized by adjusting the pH back to 7.4. Refrigeration tended to accelerate the formation of microcolloids.

Hematoporphyrin derivative is labeled with ^{99m}Tc by a pH 7.4 chemical method.[11] The labeling procedure is as follows:

1. Into a 10ml sterile, evacuated serum vial containing 0.5 ml of a solution of 0.1 mg $SnCl_2$ in 0.05 N HCl, inject 2 ml (60 mCi) ^{99m}Tc-pertechnetate in normal saline. Mix the contents of the vial for 1 minute and allow to stand at room temperature for 5-10 minutes for the complete reduction of ^{99m}Tc.
2. Adjust the pH of the radioactive mixture to 7.4 with 0.75 ml pH 12.4 sodium citrate/NaOH solution.
3. Immediately, inject 1 ml of the HPD solution into the vial slowly with continuous swirling motion for 2 minutes.
4. Incubate the contents of vial at room temperature for 30 minutes.

The binding efficiency of ^{99m}Tc-HPD was assessed by ascending radiochromatography with Whatman No. 1 paper and instant thin layer chromatography with silica gel plates (ITLC-SG)** and developed in acetone. Samples of labeled and unlabeled HPD were spotted on the chromatographic stripes measuring 1 x 11 cm, air dired and developed in a 15 ml test tube containing 1.0 ml acetone. Labeled and unlabeled HPD remained at the origin of the chromatogram in both media, whereas, free or unbound ^{99m}Tc migrated toward the solvent front with a Rf value of 1.0. While unbound and presumably ^{99m}Tc-(Sn)complex species would not migrate in Whatman No. 1 paper (Rf = 0.0), it could be separated from the labeled HPD with ITLC-SG plates (Rf = 1.0). The actual amount of free ^{99m}Tc or unbound ^{99m}Tc-(Sn)complex species was determined by analysing data from both chromatographic media. Thus, by subtracting the amount of free ^{99m}Tc obtained from Whatman No. 1 paper from the total activity found in the solvent front of the ITLC-SG plate, one could obtained

** Gelman Instrument Co., Michigan

an accurate amount of unbound ^{99m}Tc-(Sn)complex species present in the final product. Following identification of the radioactivity peaks, the chromatograms were observed for fluorescence under a UV light source.+ The Rf values obtained from fluorescent inspection were compared with the radioactive peaks. (See Table I.)

Table I. Rf values of ^{99m}Tc-HPD as determined by ascending radiochromatography with Whatman No. 1 paper and ITLC-SG plates developed in acetone and by UV light fluorescence inspection.

Radiopharmaceutical	Whatman No. 1 Paper		ITLC-SG	
	Radioactive peak*	Fluorescent peak	Radioactive peak*	Fluorescent peak
$^{99m}TcO_4^-$	1.00	-	1.00	-
^{99m}Tc-(Sn)complex species	0.00	-	1.00	-
^{99m}Tc-HPD	0.00	0.00	0.00	0.00

* = Rf values (-) = No fluorescence

Radiolabeled HPD was further analyzed by an acid precipitation method.[13] One ml of ^{99m}Tc-HPD solution was added to a test tube containing 2 ml of 0.1 N HCl. After standing at room temperature for 5 minutes, a black precipitate was formed. The precipitate was removed from the supernatant by centrifugation and washed twice thoroughly with 2 ml 0.1 N HCl. The pink-colored supernatant which contained presumably an acid soluble HPD fraction was extracted three times with equal volumes of cyclohexanone in a separatory funnel. The two phases were subsequently separated and collected in the test tubes for later radioactivity assay and fluorescent inspection.

+ UV Product, Calif.

RESULTS AND DISCUSSION

Porphyrins and related analogs are complex tetrapyrrole compounds capable of forming stable coordinated complexes with many metallic ions to form metalloporphyrins. It is in the form of metal complexes such as hemoglobin, vitamin B-12, cytochrome, catalase, peroxidase and chlorophyll that they exert their most important biological activities in the normal metabolism of plant and animal. Many of these compounds exhibit strong fluorescence when exposed to an appropriate exciting light source.

Hematoporphyrin, an artifical porphyrin compound, is prepared by treating hemoglobin with concentrated sulfuric acid. It is a crude mixture of serveal porphyrins. Hematoporphyrin derivative (HPD), a recrystallized form of hematoporphyrin, is a complex mixture of hematoporphyrin diacetate, hematoporphyrin monoacetate, vinyl porphyrins, protoporphyrin, deuteroporphyrin and several additional analogs. The principle component in HPD is hematoporphyrin diacetate.[12]

Several porphyrin compounds had been labeled with radionuclides such as ^{64}Cu and ^{57}Co. Protoporphyrin and hematoporphyrin labeled with ^{64}Cu were shown to concentrate in mouse tumors but failed to achieve significant tumor uptake in human beings.[13] Similar findings were obtained with ^{57}Co-labeled hematoporphyrin.[14] Although HPD had been used clinically as a tumor marker for various forms of neoplasms, no known radiolabeled HPD had been reported in the literature.

The present study is an attempt to labeled HPD with the radionuclide ^{99m}Tc. Of a total of 14 batches of HPD labeled with ^{99m}Tc, an average binding efficiency of 97.33% $\pm$ 1.79% was achieved as assessed by radiochromatography with Whatman No. 1 paper. Free or unbound $^{99m}TcO_4^-$ was 2.77% $\pm$ 1.82%. ITLC-SG data indicated that the final labeled product contained 91.60% $\pm$ 6.11% ^{99m}Tc-HPD, 5.63% unbound ^{99m}Tc-(Sn)complex species and less than 3% free ^{99m}Tc. Fluorescence was observed only at the origin of the chromatogram corresponding to labeled HPD. Stability determinations indicated that the labeled product remained stable at room temperature up to 3 hours. Beyond 3 hours, an increasing amount of unbound ^{99m}Tc-(Sn)complex species

was noted. Refrigeration at 2°to 8°C immediately following labeling prolonged the stability of ^{99m}Tc-HPD up to 6 hours. No evidence of microcolloids was observed by microscopic examination over a 24 hours period. However, the compound should be labeled at a pH above 7 to prevent microcolloid formation. Labeled and unlabeled HPD will precipitate out from solution when the pH of the medium drops below 6.

Acid precipitation analysis of the labeled product yielded two radioactive HPD fractions; a black precipitate, ^{99m}Tc-HPD_1, and an acid soluble fraction, ^{99m}Tc-HPD_2, which remained in the supernatant. The black precipitate, which accounted for an average of 66.37% ± 9.28% of the total radioactivity, could be redissolved in normal saline made alkaline to pH 11.5 with 1 N NaOH. However, when readjusted to pH 7.4 with 1 N HCl, the label came off with increasing amount of free ^{99m}Tc detected in the chromatogram, an indication that ^{99m}Tc-HPD_1 could not sustain repeated treatment with acid or base.

Radioanalysis of the supernatant confirmed the presence of a second radioactive HPD fraction. ^{99m}Tc-HPD_2 was extracted from the supernatant with equal volume of cyclohexanone. After separating the two phases, an average of 27.07% ± 9.41% of the radioactivity which included less than 3% free ^{99m}Tc was recovered in the fluorescent organic phase. About 6.56% ± 2.49% of the radioactivity, presumably unbound ^{99m}Tc-(Sn)complex species, remained in the non-fluorescent aqueous phase. ^{99m}Tc-HPD_1 and ^{99m}Tc-HPD_2 together accounted for an average of 90.67% ± 3.56% of the total radioactivity. (See Table II)

The chemical nature of these two radiolabeled HPD fractions has not been determined. Since HPD contains several different porphyrin analogs, some or all of these compounds may be labeled with ^{99m}Tc by the labeling process. ^{99m}Tc-HPD_1 and ^{99m}Tc-HPD_2 each may contain more than one labeled porphyrin fraction. On the other hand, the acid precipitation procedure itself may affect the labeled product and makes it appear that there two different ^{99m}Tc-labeled HPD fractions.

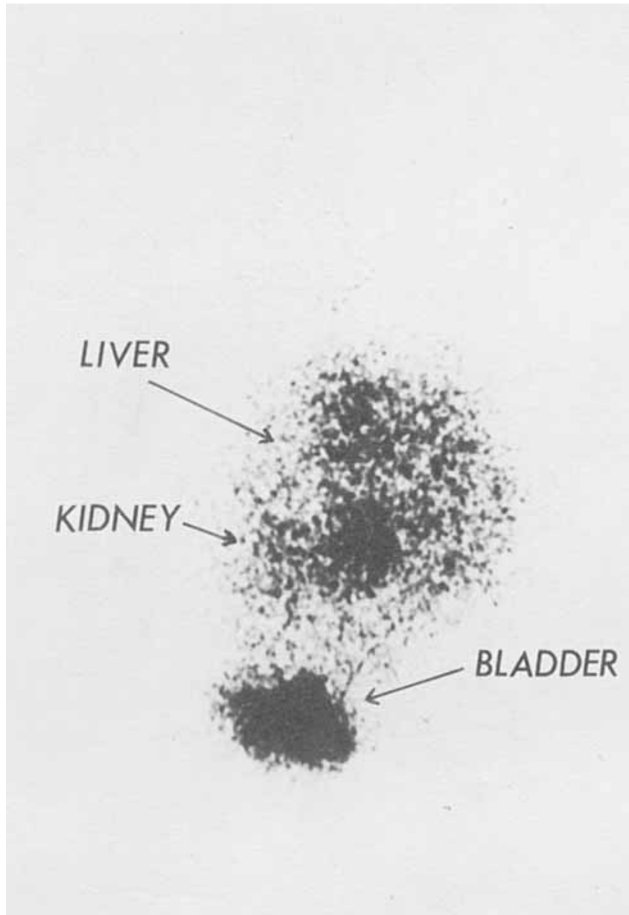

Figure 1. Anterior scintigram of a normal Swiss-Webster white mouse obtained 24 hours post intraperitoneal injection of 3 mCi of ^{99m}Tc-HPD. The animal weighing approximately 30 g was scanned under an Anger camera equipped with a pinhole collimator.

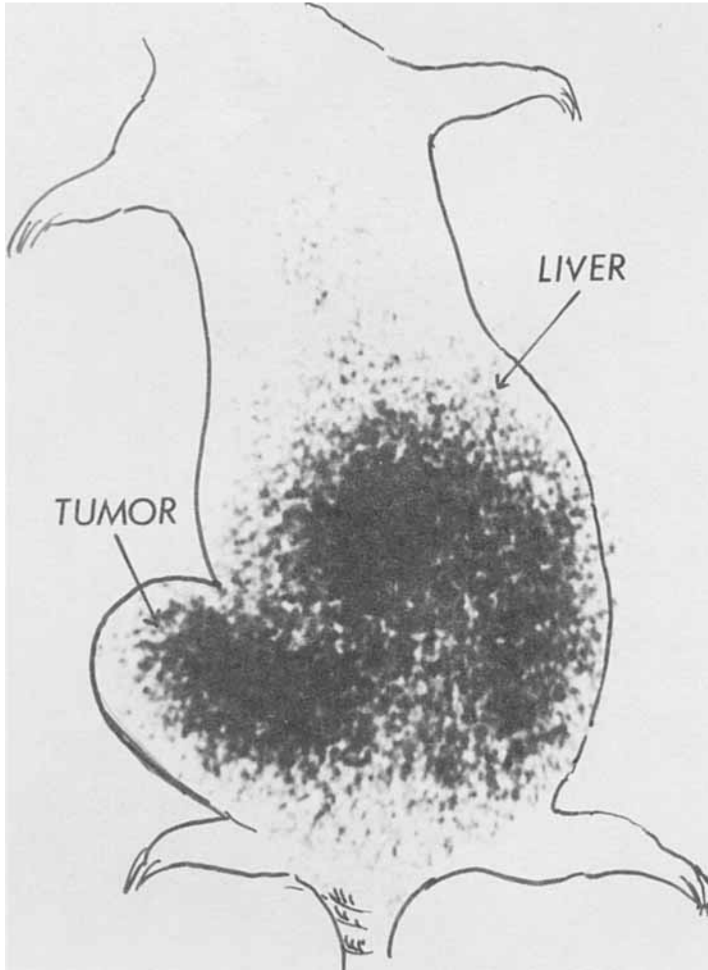

Figure 2. Anterior scintigram of a CFW strain Swiss-Webster white mouse with a large mammary adenocarcinomas. The scan is obtained 24 hours post i.p. injection of the radiopharmaceutical to allow reduction of blood pool activity.

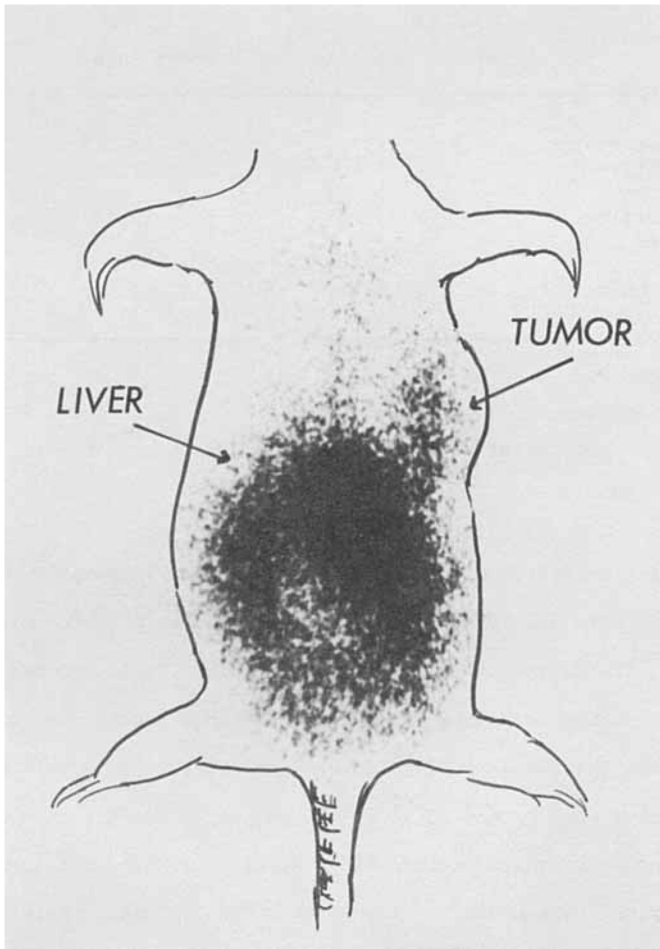

Figure 3. Anterior 24 hrs. delay scintigram of a CFW strain-Webster white mouse with a large breast tumor. Hot focal defect is seen in the scan corresponding to the anatomical site of the breast tumor.

Table II. Binding efficiency of ^{99m}Tc-HPD as determined by radiochromatography with (A) Whatman No. 1 paper and (B) ITLC-SG plate developed in acetone and by (C) acid precipitation method.

Radiopharmaceutical	(A) Percent bound^{+}	(B) Percent bound	(C) Percent bound
^{99m}Tc-Hematoporphyrin derivative	97.33 (1.79)	91.60 (6.11)	90.67 (3.56)**
^{99m}Tc-(Sn)complex species	-	5.63 (6.16)	6.56 (2.49)
^{99m}Tc-pertechnetate	2.77 (1.82)	2.77 (1.82)*	2.77 (1.82)*

+ Mean % $\pm$ (s.d.)

* Based on Whatman No. 1 paper data.

** Radioactive precipitate + cyclohexanone extracted ^{99m}Tc-HPD$_2$ fraction.

The labeling mechanism of ^{99m}Tc-HPD is not known. Presumably, incorporation of the radionuclide with HPD ligand involves the formation of a coordinated complex with reduced ^{99m}Tc. On the other hand, labeling of ^{99m}Tc to HPD may occur at the carboxylic side chains resulting in a weaker and less stable form of ^{99m}Tc-porphyrin complex. However, the recovery of ^{99m}Tc-HPD$_1$ precipitate by dilute acid strongly suggests that some stable form of ^{99m}Tc-HPD complexes exist in the labeled product. The chemical nature of these radiochemical species has not been identified.

The biological behavior of ^{99m}Tc-HPD is currently under investigation with outbred CFW^{++} strain Swiss-Webster white mice long maintained in our closed colony. These animals have a high incidence of spontaneous mammary adenocarcinomas which were confirmed by histologic examination. Preliminary results from imaging and tissue distribution studies demonstrate that radiolabeled HPD, like the parent compound, is taken up by neoplastic tissue.(See Fig. 1,2 & 3)

++ Charles River Lab. MASS.

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