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N-HYDROXYSUCCINIMIDE-HIPPURAN ESTER: APPLICATION FOR RADIOLABELING OF MACRO-MOLECULES

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A method for synthesis of N-hydroxysuccinimide ester of radioactive hippuran is developed in order to label human serum albumin in a simple and efficient manner. Organ distribution in mice and rats for the labeled albumin preparation and the commercial radioiodinated serum albumin is similar. Hippuran metabolite released from the labeled preparation into the blood stream results in its rapid urinary clearance. The hippuran labeling method offers a mild and rapid protocol for radioiodine labeling of proteins and antibodies for application in diagnostic nuclear medicine procedures.

Radioiodine labelled proteins of high specific radioactivities have wide applications in a variety of biochemical studies. Specific antibodies labeled with various radionuclides are increasingly being applied for visualizing undetectable tumors and metastases (1-4). In addition, labeled antibodies offer a means of radioimmunotherapeutic ablation of tumors for which conventional therapies are ineffective (5,6).

A variety of labeling procedures of large molecules with radioiodine have been developed over the years. The most widely used chloramine-T procedure (7) offers an efficient method for substitution of iodine into the tyrosyl residues of proteins and antibodies. However, some disadvantages of this procedure are: (i) Proteins that lack tyrosine cannot be iodinated; (ii) Oxidizing and reducing agents denature the protein to a certain extent. The rapid metabolism of radioiodinated antibodies leads to the incorporation of metabolized iodine into the thyroid and large levels of circulating radioiodine in the blood mask tumor uptake considerably (8).

In this report, the moiety, (^{125}I)-OIH is conjugated with HSA via N - Hydroxy-succinimide ester (9). The latter reacts with proteins under mild conditions in aqueous solution at neutral pH. The (^{125}I)-OIH-labeled protein offers the advantage of rapid blood clearance of the radioiodine labeled

Abbreviations Used:
OIH: Orthoiodo hippuric acid; OIH-OSU, N-Hydroxy succinimide ester of OIH; DSC, Di- (N-succinimidyl) carbonate; RISA, Radioiodinated serum albumin (commercial preparation).

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hippuran moiety upon metabolic breakdown and thus the target to background ratio is enhanced at short time intervals post injection.

MATERIALS AND METHODS

OIH, DMF, ethyl acetate, sodium azide and petroleum ether were of ACS grade purity. HSA was purified on a 21.8 x 1.8 cm Sephadex G-100 Column equilibrated with 0.01M sodium phosphate buffer (pH 7) to remove preservative materials . NMR spectra were obtained on a Varian XL200 instrument. Chemical shifts were relative to an internal standard tetramethyl silane. HPLC was carried out in an isocratic mode on DuPont 850 HPLC chromatography system using Zorbax GF 250 column with mobile phase as 0.1 M sodium phosphate buffer at pH 7 and UV detection at 280 nm.

PREPARATION OF OIH-OSU:

A mixture of a molar equivalent of cold OIH, DSC and pyridine in DMF was stirred at room temperature overnight (10). The solvent was flash evaporated and repeated crystallization of the residue from ethylacetate petroleum ether gave the OIH-OSU ester as a pure product.

Preparation of (^{125}I)-OIH was carried out as per the proceudre of Elias by silica gel thin layer chromatography using solvent system (n-butanol:glacial acetic acid: water; 6:1.5:2.5) to be more than 96%.

Preparation of the labeled ester was carried out using a mixture of labeled OIH (6 mg), DSC (5.1 mg) and pyridine (1.7 mg) in 150 ul of DMF and stirring for 4 hours at room temperature under dry conditions. The reaction mixture containing (^{125}I)-OIH-OSU ester was used without any further separation to label human serum albumin.

SYNTHESIS OF (^{125}I)-OIH-CONJUGATED ALBUMIN:

50 ul of the above reaction mixture was added to human serum albumin solution 1 ml (7 mg/ml) in 0.01 M sodium phosphate buffer (pH 7.0) mixing every 10 min for half hour. The conjugated protein was purified using 21.8 x 1.8 cm Sephadex G-100 column equilibrated with 0.01 M sodium phosphate buffer (pH 7) or dialysis at 5 C against phosphate buffer (21 x2) using spectropor membrane tubing for 3 days.

ELECTROPHORESIS:

(^{125}I)-OIH-conjugated HSA was analyzed by SDS polyacrylamide gel electrophoresis (12). Labeled albumin samples in phosphate buffer were applied to 10% acrylamide slab gel and protein fractions visualized with commassie blue stain.

ANIMAL STUDIES:

Biodistribution of the labeled protein at 60 min, 120 min and 240 min was carried out in CD-1 male adult mice (Charles River) with a dialyzed preparation (7 mg protein/ml). Labeled protein purified through a column of G-100 sephadex was used for distribution in adult male rats (Sprague Dawley). Biodistribution with commercial radioiodine labeled albumin peparation (RISA) in animals of both species was carried out for comparison with the labeled protein. The injectate consisted of 0.1 ml containing 1-3 uCi of (^{125}I) activity for all biodistribution studies.

RESULTS AND DISCUSSION

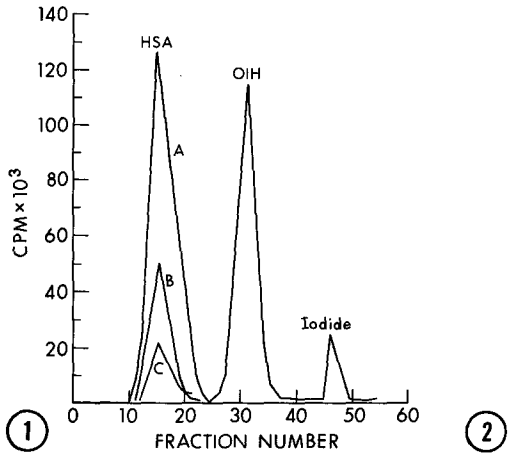
The OIH-OSU was prepared in 87% yield by a recently introduced reagent DSC used in peptide synthesis (10). A reaction time of 4 hr was enough to complete the reaction; improved yields might result with longer reaction periods with some reactions. The chemical yields are usually over 85%. The OIH-OSU active ester was isolated in pure form with M.P. 205-207 ^{o}C. Its structure was confirmed by C H N analysis and NMR spectroscopy. C H N Anal. Calcd for C_{13} H_{11} N_2 O_5 I: C,38.83; H, 2.73; N, 6.96. Found C, 38.96; H,2.62; N, 7.01. NMR (Me_2SO-d_6): 3.28 (S, 4H), 4.42 (dl, J=2.2 Hz 2H), 7.2-7.54(m, 3H), 7.94 (d, J=3.8 Hz 1H), 9.0(S,1H).

The synthesis of labeled OIH-OSU was achieved in a similar manner as the cold ester except that it was confirmed in later experiments that 2 hr reaction time gave similar yields. The reaction mixture was used directly without purification to conjugate with albumin. The impurities in the reation mixture, unreacted N-hydroxysuccinimide and OIH and free iodide being non-reactive to protein were separated by dialysis or simple gel filtration. coupling efficiency was determined to be 30 to 50% using dialysis procedure and the efficiency did not improve with prolonged reaction time. There was no change in initial and final pH of reaction mixture. HPLC analysis of the contents of the dialysis bag contents gave a single radioactive peak at T_r=8.59 min. RISA and unlabeled albumin gave retention times of 8.56 min and 8.69 min respectively.

Gel filtration on G-100 sephadex column using 0.01 sodium phosphate buffer (pH7) of the labeled albumin gave the elution profile shown in Figure 1 indicating the separate peaks for the protein, free hippuric acid and free iodide. The three day dialyzed preparation showed the albumin activity at the same peak volume. The elution profile of commercial RISA was in close agreement with that of coupled protein. Incorporation of 38-50% of the radioactivity (mean 44.3%) was found in the conjugate in three preparations when the protein was purified through column. The SDS gel electrophoresis for the preparations is shown in Figure 2.

The biodistribution data for the labeled albumin preparation along with the commercial RISA are given in Tables 1 and 2. These data show that (^{125}I)-OIH conjugated albumin has a biodistribution identical to that of commercial radioiodinated albumin. At 4 hr post administration, however, urinary excretion of radioactivity was almost three times that of commercial albumin and there was no accumulation of activity in thyroid. In the case of OIH coupled albumin, rat urine analysis by ITLC and HPLC showed that the activity corresponded to (^{125}I)-OIH.

The results presented indicate that the proposed method may be used for the preparation of iodine labelled proteins having similar properties to those



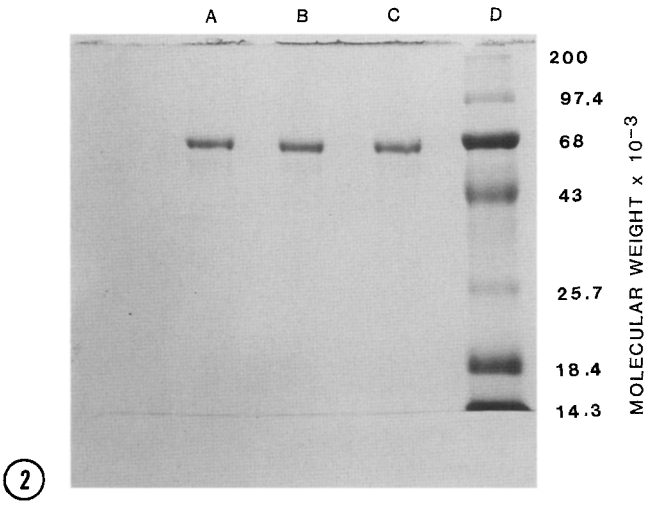


Figure 1. Elution profiles of OIH Labeled HSA and RISA:
Curve A: OIH-OSU and HSA reacted for one half hour, loaded on Sephadex G-100 column and eluted with phosphate buffer at pH 7; curve B: Dialysed OIH-OSU; Curve C: Commercial RISA

Figure 2. SDS-polyacrylamide gel electrophoresis patterns on 10% polyacrylamide gel (12): A. OIH-HSA; B: HSA; C. RISA: Marker proteins: myosin (200 K), phoshorylase b (97.4 K), bovine serum albumin (68 K), ovalbumin (43 K), alpha-chymotripsinogen (25.7 K), beta- lactoglobulin (18.4 K) and lysozyme (14.3 K)

prepared by direct radioiodination. The proposed procedure involves no harsh chemical environment during labeling. The techniques available for achieving high specific activity OIH allows the incorporation of a large amount of radioactivity on the protein even though a given protein may be modified at only a single site. The synthesis of OIH-OSU active ester was achieved in

TABLE 1

BIODISTRIBUTION OF ^{125}I-OIH CONJUGATED HUMAN SERUM ALBUMIN IN MICE*(%DOSE/ORGAN)@

	1 HOUR		4 HOUR		24 HOUR	
ORGAN	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL
Blood	59.1±7.1	66.1±5.2	51.0±5.8	52.3±1.9	18.6±2.4	23.1±1.7
Bone	5.7±0.8	7.8±0.4	5.5±1.1	5.5±1.2	3.0±0.5	1.9±0.5
Kidney	3.9±0.5	6.2±0.8	3.3±0.7	4.4±0.5	1.5±0.4	1.6±0.3
Urine	0.7±0.4	6.0±1.5	3.9±1.0	17.8±2.0	-	-
Heart	0.9±0.2	0.7±0.1	0.9±0.2	0.7±0.1	0.3±0.1	0.2±0.0
Lungs	1.8±0.7	1.5±0.5	1.5±0.3	1.5±0.8	0.8±0.2	0.5±0.1
Liver	6.9±0.8	4.6±1.6	5.6±0.7	6.4±0.7	2.7±0.7	2.4±0.3
Muscle	6.9±1.4	7.9±1.8	6.9±1.4	11.5±1.2	8.3±0.7	5.4±0.5
Spleen	0.4±0.1	0.6±0.6	0.3±0.1	0.3±0.0	0.1±0.1	0.1±0.0
Thyroid	-	-	1.5±0.2	-		

* # of Animals = 6; @ Mean ± 1 S.D.; ^{125}I-RISA used as CONTROL.

TABLE 2

BIODISTRIBUTION OF ^{125}I-OIH CONJUGATED HUMAN SERUM ALBUMIN IN RATS*(% DOSE/ORGAN)@

	1 HOUR		4 HOUR		24 HOUR	
ORGAN	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL	CONTROL	EXPERIMENTAL
Blood	69.5±3.6	64.0±4.4	51.8±3.4	52.0±4.3	16.6±0.9	14.4±0.9
Bone	9.6±.3	9.3±1.6	6.7±2.9	6.8±0.05	4.2±0.5	1.1±0.08
Kidney	2.4±0.1	3.2±0.3	1.9±0.2	3.7±1.3	0.6±0.1	0.2±0.0
Urine	0.8±0.2	4.0±0.4	1.4±0.8	7.6±2.7	-	-
Heart	0.9±0.2	0.8±0.1	0.7±0.1	0.7±0.2	0.3±0.1	0.1±0.0
Lungs	1.4±0.1	1.6±0.2	1.3±0.2	1.1±0.1	0.5±0.1	0.1±0.0
Liver	9.2±1.6	10.7±1.1	4.6±1.0	5.4±0.5	1.8±0.2	0.6±0.08
Muscle	9.5±1.9	9.2±0.8	12.5±2.7	9.7±1.2	10.2±0.9	2.0±0.3
Spleen	0.3±0.1	0.4±0.1	0.2±0.0	0.3±0.0	0.1±0.0	-
Thyroid	0.2±0.1	-	0.3±0.1	-	0.1±0.1	-

* # of Animals = 6; @ Mean ± 1 S.D.; ^{125}I-RISA used as CONTROL.

good yield using DSC. Using this agent to form the active ester avoids cumbersome process of using dicyclo hexyl-carbodiimide and separating insoluble urea from the reaction mixture. Labeled N-hydroxysuccinimide ester could be coupled to protein in aqueous solution at room temperature and produce an amide bond with the amino group of lysine residue of protein.

In this report, human serum albumin was used as a model system to test the incorporation of the labeling moiety via the succinimide ester in a simple manner. The reaction proceeds rapidly with good yields under gentle conditions. The SDS gel electrophoresis shows no indication of degradation or aggregation of the protein molecule. Labeled protein could be purified by dialysis or by G-100 Sephadex column readily.

Biodistribution experiments in animals show an identical behavior between the OIH labeled albumin and (^{125}I)-RISA. The synthetic procedure did not seem to bring about any change in the protein molecule. Upon metabolic breakdown of the OIH-Albumin, the hippuran moiety is eliminated rapidly from the body via urinary tract. This is definite advantage over the iodinated albumin which upon deiodination, leaves the iodide largely in circulation for prolonged periods giving rise to a large body background besides accumulating in the thyroid gland.

The present study thus offers a viable method for radioiodo labeling of proteins and antibodies for application in diagnostic nuclear medicine procedures for imaging without the annoying background of free circulating iodide in blood pool for prolonged periods. The labeled ester is quite stable and can be supplied in a kit form for ready labeling with any large molecule.

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