INDIRECT LABELING OF PROTEINS WITH RADIOIODINE

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ABSTRACT

A procedure is described for the radioiodination of proteins using an iodinated derivative of N-succinimidyl 3-(tri-n-butylstannyl)benzoate (ATE), previously described by Zalutsky et al (6). ATE was obtained in a high pure form and the iodination has been performed with 131-Iodine in 70-80% yield. Protein labeling studies performed with human IgG indicate that the ATE intermediate is an important alternative to conventional labeling methods.

I. INTRODUCTION

Direct radioiodination is the method which has been most frequently employed for labeling proteins with high specific activity.

Many scientific investigations have been carried out using eletrophilic iodination method with Chloramine-T [1] and Iodogen [2] as oxidant agents. It has been noted that even under mild oxidizing conditions some proteins lose a portion of their biologic activity, most likely from oxidation of thiols or from iodination of a tyrosine that is involved in a specific interation such as binding [3].

However, the principal problem with the use of radioiodinated proteins is the in vivo dehalogenation, presumably related to the structural similarity between the iodophenyl group and thyroid hormones.

Some prosthetic groups developed for indirect labeling of proteins as Bolton and Hunter (N-succinimidyl para-hydroxyphenylproprionate) [4] and Wood's reagent (para-hydroxybenzimidate) [5], do not appear to alter biologic activity of the labeled proteins, but the in vivo use of these reagents has been found to be limited in some cases as a result of in vivo deiodination probably do to the substitution of the iodine ortho to a hydroxyl group on an aromatic ring as in the direct labeling.

Studies developed by Zalutsky et al. [6] and Wilbur et al [3] have demonstrated that antibodies can be radioiodinated using the N-succinimidyl-(tri-n-butylstannyl)benzoate intermediate (ATE) and that the use of ATE reagent for protein labeling significantly reduced the thyroid uptake of radioiodine.

We reproduced the ATE synthesis as described by Zalutsky et al [6] and we studied the labeling of this precursor with radioiodine using 131-Iodine. We also studied the use of this derivative on labeling human IgG as a protein model. These studies are part from an IAEA Co-ordinated Research Program on Optimization of Synthesis and Quality Control Procedures for the preparation of ^{18}F and ^{123}I labeled peptides.

II. MATERIAL AND METHODS

Materials

All reagents were purchased from Sigma-Aldrich. Solvents used were HPLC grade. Sep-Pack silica cartridges were purchased from Waters, the HPLC column (Partisil 10 4.6x250 mm) were purchased from Whatman and Sephadex G25 pre-packed PD-10 column from Pharmacia. Human IgG were purchased from Sandoz. 131-Iodine were obtained from Ipen-Cnen/Sp in NaOH solution and diluted in PBS pH 7.

Methods

Synthesis of ATE - ATE was synthesized from m-bromobenzoic acid as described by Zalutsky et al [6]:

- Preparation of tri-n-butyl tin 3-(tri-n-butylstannyl)-benzoate. Under reduced temperature (-95 to -100^{o}C), in tetrahydrofuran (THF) medium, m-bromobenzoic acid reacted with n-butyllithium in hexane followed by the quenching the dilithio-anion with tri-n-butyl tin chloride. The product was extracted in ether and purified by flash chromatography.

- Synthesis of N-succinimidyl 3-(tri-n-butylstannyl)benzoate (ATE). The stannyl ester obtained above reacted with N-hydroxysuccinimide (NHS) and dicyclohexylcarbodiimide (DCC) in dry THF for 12 hours at room temperature. The reaction mixture was filtered to remove dicyclohexylurea and the product was purified by flash cromathography. The final product was analyzed by IR and the spectrum compared with IR spectrum from a sample of ATE supplied by Dr. Zalutsky as well as the comparison of HPLC profiles using a Silica column (10μm) and hexane:ethyl acetate:acetic acid (70:30:0.2) as solvent.

Labeling ATE with 131-Iodine

Some stock solutions were prepared to study different labeling parameters:

(a) NaI 1M in NaOH 0.01N
(b) Acetic Acid 1M in chloroform
(c) t-butylhydroperoxide (TBHP) 1M in chloroform and Na_2SO_4 to remove water
(d) ATE 0,1M in chloroform

A cold reaction was conducted in a little conical vial: 8 μL (a) + 8 μL (b) + 8 μL (c) + 20 μL (d).

The reaction mixture was stirred at room temperature. TLC silica gel chromatography was performed in 30% ethyl acetate in hexane and showed two spots at Rf 0.48 and 0.24 as described by Zalutsky et al (6) that corresponds to ATE and the iodinated derivative respectively.

Radioiodination studies were performed using 3.7-7.4 MBq (100-200μCi) of $Na^{131}I$ pH 7 (diluted in PBS). In a 3 mL conical vial were added 50μL of solutions (b) and (c) and 10μL of solution (d), prepared as described above. The percent of the radioiodinated precursor was determined by the same TLC chromatographyc system described above.

The reaction mixture was stirred at room temperature for 30 min and ^{131}I-labeled was isolated by chromatography over a silica gel Sep-Pak cartridge. The column was first saturated with hexane and the reaction mixture was loaded on the column with the help of 300μL of hexane. Following elution with 40mL of hexane and 25 mL of 8% ethyl acetate in hexane, the product was isolated in approximately 18 mL of 30% ethyl acetate in hexane.

To this original labeling conditions proposed by Zalutsk et al [6], we studied the influence of TBHP concentration varying the volume of the stock solution from 12.5 to 100μL and we also studied the influence of the pH on labeling yield.

Radioiodination of protein using the ^{131}I-intermediate

The 30% ethyl acetate eluate containing N-succinimidyl-3-[^{131}I]iodobenzoate was evaporated to 1 mL, transferred to a 1ml-connical vial and evaporated to dryness with a stream of nitrogen. Human IgG (500μg/50μL of 100mM borate buffer, pH 8.5) was added and the mixture incubated in ice on a shaker for 30 min. The reaction was terminated by the addition of 300μL of 200mM glycine in borate buffer followed by the incubation of 5 min at room temperature. The ^{131}I-labeled IgG was separated from other reaction products using a Sephadex G-25 column eluted with PBS.

Radiochemical purity was determined by ITLC-SG chromatography using methanol 85% as solvent (Rf = 0 to iodinated IgG and Rf = 1 to free radioiodine).

Radioiodination of protein using Iodogen method

Iodogen tubes were prepared as described by Salacinsky et al [7]: Iodogen (1mg) was dissolved in dichloromethane (1 mL) and 10 μL of this solution was dispersed in the bottom of a polypropylene iodination vial and evaporated to dryness at room temperature under nitrogen stream.

Human IgG 0.5M solution was prepared in phosphate buffer pH 7.5 (100μg/10μL) and was added to the Iodogen tube followed by $Na^{131}I$ solution (3.7MBq, 30-50μL in NaOH 10^{-3}M). The iodination was allowed to proceed for 30 minutes at room temperature with gentle stirring and it was finished by decanting the mixture from the reaction vial.

Purification of labeled IgG and radiochemical purity determination were accomplished as previously described in the ATE labeling method.

Biological distribution studies

Biological distribution studies were developed in normal mice, using the IgG labeled by direct and indirect methods, in order to compare the in vivo dehalogenation of the protein. Radiopharmaceuticals were administered by the tail vein (0.74 - 1.48MBq/dose) and the % administered dose/organ were determined at 1, 4 and 24 hours after the dose administration.

III – RESULTS AND DISCUSSION

The iodinated precursor N-succinimidyl 3-(tri-n-butylstannyl)benzoate (ATE) was synthesized as described by Zalutsky et al [6] and obtained in a pure form as observed in the IR spectrum (Figure 1 – ATE-IPEN) compared with the IR spectrum of a standard sample supplied by Dr Zalutsky laboratory (Figure 1 – ATE-STD).

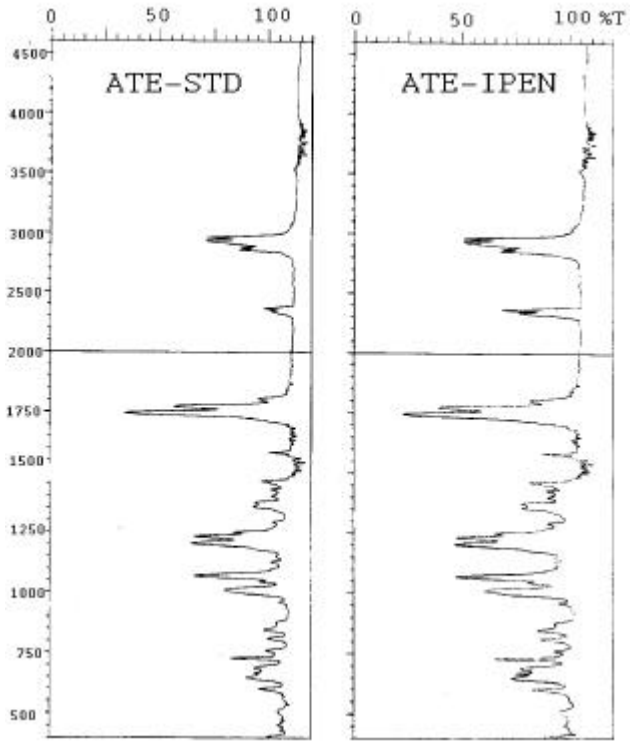

Figure 1. IR Spectrum of synthesized ATE (ATE-IPEN) and ATE standard sample (ATE-SND).

Iodination of ATE was performed with $Na^{131}I$ and the radiochemical purity vary from 72.22 ± 5.50 at pH 3.0 – 4.0 to 69.48 ± 4.15 at pH 5.0- 5.5 (means of 5 determinations to each case).

The purification on Sep-Pack system indicated that about 40% of the initial radioactivity are recuperated in the 30% ethyl acetate in hexane fraction. Labeling conditions using pH 3.0-4.0 indicate that the percent of radioactivity remained in Sep-Pack after purification is relatively lower (about 13%) when compared with labeling at pH 5.0-5.5 (about 30%). The better radioactivity loss in the first case is on 8% ethyl acetate in hexane fraction (about 25%).

The percent of iodinated precursor depends of the TBHP concentration as observed in Figure 2, on labeling conditions using pH 5.0-5.5 and different volumes of 1M TBHP solution.

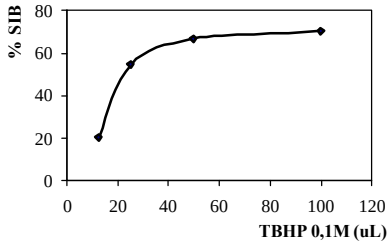

Figure 2. % of Iodinated precursor X TBHP

Zalutsky et al [6] observed the pH dependence on protein radioiodination using the radioiodinated precursor and concluded that the optimal coupling efficiencies are obtained under mildly alkaline conditions, so we use in our studies the pH 8.5 on protein coupling reaction. The same study suggest that the efficiency of protein labeling using radioiodinated ATE is also dependent on the protein concentration so we used in our studies 500µg of protein.

After Sephadex G-25 purification step, about 40% of the initial activity (radioiodinated ATE) are isolated as labeled protein.

Direct labeling of IgG using Iodogen method showed a radiochemical purity of 80-90% determined by ITLC-SG.

The results from biological distribution studies were summarized in Tables 1 and 2.

TABLE 1. Percent administered dose/organ in normal mice of radioiodinated IgG labeled by direct method

ORGAN	% ADMINISTERED DOSE/ORGAN		
	1 hour	4 hours	24 hours
total blood	13.99 ± 1.31	6.68 ± 0.12	2.37 ± 1.15
kidneys	4.84 ± 0.06	2.95 ± 0.29	1.89 ± 0.88
liver	68.42 ± 5.00	40.54 ± 3.88	4.20 ± 0.89
heart	0.39 ± 0.07	0.20 ± 0.05	0.08 ± 0.04
spleen	2.53 ± 0.29	1.05 ± 0.25	0.43 ± 0.19
stomach	12.18 ± 0.70	8.56 ± 2054	0.95 ± 0.70
lung	1.77 ± 0.90	0.71 ± 0.36	0.14 ± 0.03
thyroid	1.15 ± 0.06	4.58 ± 3.20	11.50 ± 7.97
intestines	14.17 ± 1.42	17.69 ± 3.74	4.24 ± 2.31

TABLE 2. Percent administered dose/organ in normal mice of radioiodinated IgG labeled by indirect method (ATE prosthetic group)

ORGAN	% ADMINISTERED DOSE/ORGAN		
	1 hour	4 hours	24 hours
total blood	-	14.75 ± 0.32	6.21 ± 0.57
kidneys	12.82 ± 0.44	0.63 ± 0.01	1.00 ± 0.05
liver	49.04 ± 4.53	2.32 ± 0.33	1.00 ± 0.05
heart	3.42 ± 0.10	0.18 ± 0.01	0.27 ± 0.01
spleen	2.65 ± 0.05	0.12 ± 0.02	0.06 ± 0.01
stomach	2.82 ± 0.40	0.20 ± 0.01	0.11 ± 0.01
lung	16.35 ± 2.41	0.41 ± 0.10	0.19 ± 0.04
thyroid	0.03 ± 0.00	0.06 ± 0.03	0.03 ± 0.01
intestines	19.64 ± 0.11	1.94 ± 0.15	0.78 ± 0.12

The results from biological distribution studies showed that using direct labeling protein method, the percent administered dose present on thyroid and stomach are greater, what suggest the presence of free iodine in high level when compared with indirect labeling method.

So, we concluded that IgG labeled with radioiodine using the prosthetic group (ATE), results in a protein with reduced dehalogenation in vivo.

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