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# Optimization of a convenient route to produce *N*-succinimidyl 4-radiodobenzoate for radioiodination of proteins

E.B. Araujo\*, J.S. Santos, M.T. Colturato, E. Muramoto, C.P.G. Silva

*Centro de Radiofarmácia do Instituto de Pesquisas Energéticas e Nucleares, IPEN-CNEN/ISP, Rua do Lago, Travessa R 400, Cidade Universitária, São Paulo CEP 05508-900, Brazil*

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## Abstract

The preparation of *N*-succinimidyl-4- $^{131}\text{I}$ iodobenzoate (SIB) has been optimized using an alternative technique employing Cu(I)-assisted radioiododebromination that produces *p*- $^{131}\text{I}$ iodobenzoic acid. The reaction conditions were optimized and radiochemical purity of more than 90% was obtained when using 160°C, 60 min reaction time and a [CuCl]/[*p*-bromobenzoic acid] relation of about  $10^{-2}$ . After purification, the *p*- $^{131}\text{I}$ iodobenzoic acid reacted with TSTU to produce the SIB in a radiochemical yield greater than 98%. Protein conjugation using SIB resulted in a relatively low radiochemical yield. Biological distribution studies evidenced the *in vivo* stability of the labeled protein.

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**Keywords:**  $^{131}\text{I}$ ; Proteins; Conjugation; Prosthetic group; *In vivo* stability

## 1. Introduction

The potential of using radioiodine ( $^{123}\text{I}$  and  $^{131}\text{I}$ ) labeled-peptides and monoclonal antibodies for diagnosis and treatment of tumors has created a renewed interest in the utilization of radioiodinated proteins. Radioiodination of peptides and proteins is well established (Wilbur, 1992) and the most common procedure is the *in situ* generation of electrophilic radioiodine from radioiodide by suitable oxidants such as Chloramin-T, Iodogen or enzymatically by lactoperoxidase. This direct labeling approach involves substitution of the iodine ortho to a hydroxyl group on an aromatic ring (tyrosine residue of the protein).

One of the major problems in the *in vivo* use of radioiodinated proteins is the rapid deiodination by the action of specific enzymes, probably because of the structural similarity between these iodophenyl groups and thyroid hormones (Zalutsky and Narula, 1987).

An alternative approach for protein radioiodination is prosthetic group labeling, which allows radioiodination of compounds not suitable for direct labeling. A proper choice of the prosthetic group also allows improvement of the stability against *in vivo* enzymatic deiodination. In general, these prosthetic groups are *N*-succinimidyl esters of aryl derivatives, with alkyl stannyls as leaving group in an electrophilic substitution reaction with radiohalogens. Subsequent conjugations with the protein involves nucleophilic substitution of the *N*-succinimidyl ester with free amines, e.g. lysine. After radioiodination and conjugation to proteins, these labeling reagents provide products with greater stability when used *in vivo* than the conventionally radioiodinated proteins (Zalutsky and Narula, 1987; Garg et al., 1989a, b; Lindgren et al., 1998).

The preparation of *N*-succinimidyl 3- and 4-radiodobenzoate (SIB) can be accomplished with good radiochemical yields via iododestannylation. The disadvantage of this method is the laborious synthesis of the tin precursor which require purification steps with poor yields. In this method, purification is also essential to remove the unlabeled precursor, which could compete

\*Corresponding author. Fax: +55-11-38169257.

E-mail address: [ebaraujo@net.ipen.br](mailto:ebaraujo@net.ipen.br) (E.B. Araujo).

with the radioiodinated intermediate for amine sites on the protein during the conjugation reaction (Zalutsky and Narula, 1987; Wilbur et al., 1989; Kozirowski et al., 1998).

An alternative technique to produce the SIB prosthetic group has been described using Cu(I) assisted radioiododebromination (Moerlein, 1990) to produce radioiodobenzoic acid that can then be reacted with *O*-(*N*-succinimidyl)-*N,N,N',N'*-tetramethyluronium (TSTU) in alkaline means to generate the correspondent SIB (Wester et al., 1995). In this study we describe the optimization of labeling conditions of this alternative technique to produce *N*-succinimidyl 4-[<sup>131</sup>I]iodobenzoate to be used in the routine production of labeled proteins with radioiodine.

## 2. Materials and methods

Dimethylsulfoxide (DMSO), *p*-bromobenzoic acid 99%, Copper(I) chloride (CuCl, 99%), hexane, ethyl acetate and acetonitrile (HPLC grade) and 1,3,4,6-tetrachloro-3 $\alpha$ -6 $\alpha$ -diphenylglycouril (Iodogen) were purchased from Sigma-Aldrich. TSTU was purchased from Fluka. [<sup>131</sup>I]NaI solution was obtained from Nordion (Canada) in pH 11 sodium hydroxide and processed at IPEN-CNEN (São Paulo, Brazil). Human Immunoglobulin (IgG) was supplied by Novartis Pharma.

The radioiodinated *N*-succinimidyl-4-[<sup>131</sup>I]iodobenzoate prosthetic group was obtained in a two step procedure as illustrated in the Fig. 1.

### 2.1. Iododebromination Cu(I) assisted

The iododebromination step was based on the results reported by Moerlein (1990) who investigated the utility of cuprous chloride-promoted aromatic iododebromination as a non-carrier added labeling technique for

radioiodination of aromatic compounds in high specific activity. The radioiodination experiments were performed in tightly sealed 2 ml glass vessels heated to selected temperatures with the use of a copper heating block. The *p*-bromobenzoic acid was placed in the reaction vessel (50  $\mu$ l of 0.2 M solution in DMSO) followed by the addition of CuCl (100  $\mu$ l of specific solution in DMSO) and of 5  $\mu$ l of [<sup>131</sup>I]NaI solution (3.7 MBq). We investigated the influence of temperature, reaction time and [CuCl]/[*p*-bromobenzoic acid ratio in the final yield.

Radiochemical purity of the labeling mixtures were determined by chromatographic method using Whatmann 3MM paper and chloroform:acetic acid (9:1) as solvent. In this system, the  $R_f$  value of the *p*-radioiodobenzoic acid is 1.0 and radioiodine is 0.0. The product was purified by a Sep-Pack C-18 cartridge (Waters) previously saturated and washed with 15 ml of distilled water, dried with a nitrogen stream and eluted with 5 ml of acetonitrile using a peristaltic pump with a flow rate of 10 ml/min. The unreacted radioiodine was removed in the aqueous fraction and the *p*-radioiodobenzoic acid/*p*-bromobenzoic acid in the first 2 ml of acetonitrile. This final volume was transferred to another reaction vial and reduced to 400–600  $\mu$ l using a nitrogen stream.

The HPLC elution profile of the purified product was conducted with a HPLC system (CG Scientific Instruments), equipped with a radioisotope detector (Berthold) and a variable wave length UV spectrophotometer set at 254 nm, using a Waters silica gel column (10  $\mu$ m, 250 mm  $\times$  4.6 mm) eluted with hexane:ethyl acetate:acetic acid (70:30:0.2) at a flow rate of 1 ml/min.

### 2.2. Preparation of the *N*-succinimidyl-4-iodobenzoate

To the reaction vial containing the purified *p*-radioiodobenzoic acid, 10  $\mu$ l of 2 N NaOH was added

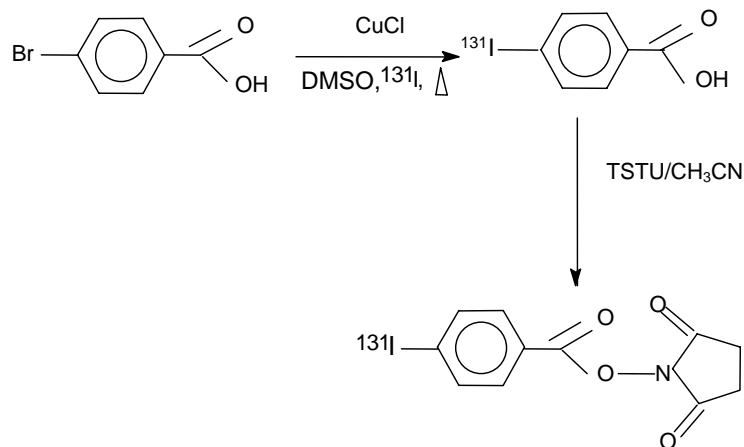


Fig. 1. Synthetic approach for *N*-succinimidyl-4-[<sup>131</sup>I]iodobenzoate preparation.

followed by 100  $\mu\text{l}$  of TSTU 0.2 M in acetonitrile. The reaction mixture was heated at 60°C for 15 min. The *N*-succinimidyl-4-[ $^{131}\text{I}$ ]iodobenzoate was purified using a silica gel Sep-pack cartridge (Waters) previously saturated with hexane and eluted with hexane followed by hexane:ethyl acetate (70:30). The product was eluted in the first 5 ml of the hexane:ethyl acetate eluent. The reaction mixture was analyzed by HPLC before and after purification procedure using the same system previously described. The HPLC profile of the purified *N*-succinimidyl-4-[ $^{131}\text{I}$ ] iodobenzoate was compared with the same product obtained by the iodination of the tri-*n*-butylstannyl derivative obtained according to the synthetic approach described by Zalutsky and Narula (1987). The product was characterized by IR and HPLC and compared with a standard kindly supplied by Zalutsky.

### 2.3. Protein conjugation

The *N*-succinimidyl-4-[ $^{131}\text{I}$ ]iodobenzoate was placed in a reaction vial and the solvent was evaporated to dryness with a stream of nitrogen. Human IgG (200  $\mu\text{g}$ /50  $\mu\text{l}$  of borate buffer pH 8.5) was added to the reaction vial and the reaction proceeded for 30 min at room temperature with gentle stirring. The reaction was terminated by addition of 300  $\mu\text{l}$  of 0.2 M glycine in borate buffer pH 8.5 followed by incubation of 5 min at room temperature.

Human IgG was also labeled by direct method as described by Salacinsky (1981) using 10  $\mu\text{g}$  of Iodogen, 100  $\mu\text{g}$  IgG in 10  $\mu\text{l}$  of 0.5 M phosphate buffer pH 7.5 and 30–50  $\mu\text{l}$  of [ $^{131}\text{I}$ ]NaI in NaOH  $10^{-3}$  M (3.7 MBq). The iodination was allowed to proceed for 30 min at room temperature with gentle stirring. Radiochemical purity of labeled proteins was obtained by chromatography using the ITLC-SG and methanol 85% as solvent. The proteins labeled by prosthetic group and Iodogen methods were purified by a Sephadex G25 gel column (5 cm  $\times$  1 cm) eluted with PBS.

### 2.4. Biodistribution studies

The biodistribution studies were performed using Swiss female mice with turpentine-promoted inflammation focus developed on the right thigh, 24 h before the radiopharmaceutical administration. A group of 20 animals received 0.37–1.11 MBq/100  $\mu\text{l}$  of IgG labeled with [ $^{131}\text{I}$ ] SIB intravenously via tail vein. Another group of 20 animals received the IgG labeled by Iodogen method. Blood samples were collected by an orbital bleed 4 and 24 h after the dose administration and the animals were sacrificed, the tissues of interest removed, washed, weighed and counted for  $^{131}\text{I}$  activity, using a gamma counter. The percent injected dose for each tissue was calculated by comparing the activity in each

tissue to injection standards of suitable count rate. (All biodistribution studies were carried out in compliance with the national laws related to the conduct of animal experimentation.)

## 3. Results

The effect of reaction temperature on the iodobromination of the *p*-bromobenzoic acid are illustrated in Table 1. The effect of the reaction time was studied in two different temperatures as showed on Table 2. Radiochemical yield was strictly dependent on the CuCl concentration with better results obtained when using [CuCl]/[*p*-bromobenzoic acid] of  $10^{-2}$  (Table 3, Fig. 2). After reaction of the *p*-radioiodobenzoic acid with TSTU, the [ $^{131}\text{I}$ ]SIB prosthetic group was obtained in a radiochemical purity of  $87.52 \pm 2.47\%$  ( $N=6$ ) analyzed by HPLC (Fig. 3). When the NaOH solution (10  $\mu\text{l}$ , 2 N) was first introduced in the reaction vial and evaporated to dryness before the introduction of the TSTU, the radiochemical purity increased to  $98.19 \pm 1.14\%$  ( $N=6$ ). In this last case, the final product was used in the protein labeling procedure without purification. The radioactive HPLC profile of this last reaction mixture (Fig. 4) presents one peak with

Table 1  
Radiochemical yield of *p*-[ $^{131}\text{I}$ ]iodobenzoic acid: influence of reaction temperature

Temperature (°C)	<i>p</i> -[ $^{131}\text{I}$ ]iodobenzoic acid (%)	Radioiodine (%)	Other impurity (%)
155	$81.59 \pm 1.26$	$11.65 \pm 1.14$	$6.75 \pm 0.13$
165	$79.48 \pm 5.03$	$20.52 \pm 5.03$	0
175	$37.33 \pm 8.82$	$42.68 \pm 7.69$	$19.99 \pm 4.07$
$N = 3$			

Reaction conditions: CuCl (100  $\mu\text{l}$ , 0.1 M); *p*-bromobenzoic acid (50  $\mu\text{l}$ , 0.2 M/DMSO); 5  $\mu\text{l}$  Na $^{131}\text{I}$  (0.37 MBq);  $t = 60$  min.

Table 2  
Radiochemical yield of *p*-[ $^{131}\text{I}$ ]iodobenzoic acid: influence of time reaction

Reaction time (min)	<i>p</i> -[ $^{131}\text{I}$ ]iodobenzoic acid (%)	
	175°C	165°C
15	$49.78 \pm 8.16$	—
30	$71.61 \pm 4.69$	$68.28 \pm 0.26$
45	$45.92 \pm 2.07$	$72.94 \pm 3.49$
60	$37.33 \pm 8.82$	$79.48 \pm 5.03$
$N = 3$		

Reaction conditions: CuCl (100  $\mu\text{l}$ , 0.1 M/DMSO); *p*-bromobenzoic acid (50  $\mu\text{l}$ , 0.2 M/DMSO); 5  $\mu\text{l}$  Na $^{131}\text{I}$  (0.37 MBq).

Table 3  
Radiochemical yield of  $p$ - $^{131}\text{I}$ iodobenzoic acid: influence of [CuCl]/ $p$ -bromobenzoic acid] relation

[CuCl]/ $p$ -bromobenzoic acid]	$p$ - $^{131}\text{I}$ iodobenzoic acid (%)
$10^{-1}$	$82.57 \pm 2.25$
$10^{-2}$	$92.73 \pm 1.51$
$10^{-3}$	$8.25 \pm 1.41$
	$N = 6$

Reaction conditions:  $T = 165^\circ\text{C}$ ;  $t = 60$  min;  $5 \mu\text{l Na}^{131}\text{I} = 0.37$  MBq.

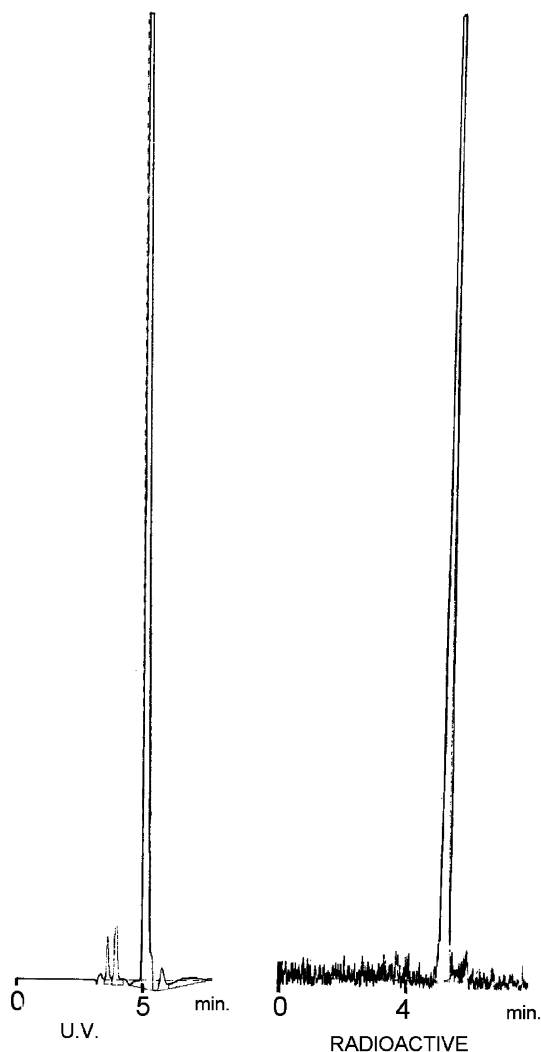


Fig. 2. HPLC profile of  $N$ -succinimidyl-4- $^{131}\text{I}$ iodobenzoate; silica column,  $10 \mu\text{m}$ ,  $4.6 \times 250 \text{mm}^2$ ; hexane:ethylacetate:acetic acid (70:30:0.2); flux: 1 ml/min.

$R_t = 10.5$  min that corresponds to the radioiodinated prosthetic group as confirmed by HPLC analysis of the correspondent iodinated tri- $n$ -butylstannyl derivative

prepared according to the synthetic approach described by Zalutsky and Narula (1987). The  $R_t$  of the  $p$ -radioiodobenzoic acid in this chromatographic system is 5.15 min.

The radiochemical yield of IgG labeled by Iodogen method was  $85.02 \pm 1.19\%$  ( $N = 5$ ) and using the indirect labeling approach with  $^{131}\text{I}$  SIB was  $15.5 \pm 5.3\%$  ( $N = 10$ ). In order to increase the radiochemical yield using the indirect approach, some labeling conditions were studied such as protein concentration (200–1000  $\mu\text{g}$ ) and reaction time (15–60 min). The solvent mixture was also evaluated using borate buffer pH 8.5 and mixtures of borate buffer:DMF:acetonitrile and borate buffer:acetonitrile. Despite all labeling conditions employed, the radiochemical yield of the IgG remained low. Fig. 5 shows the results obtained in biological distribution studies using IgG labeled via  $^{131}\text{I}$ SIB and IgG labeled by Iodogen method.

#### 4. Discussion

The synthetic approach used to produce the  $N$ -succinimidyl 4- $^{131}\text{I}$ iodobenzoate prosthetic group here described was originally used in the production of the Fluorine-18 derivative  $N$ -succinimidyl 4- $^{18}\text{F}$ fluorobenzoate (Vaidyanathan and Zalutsky, 1992; Guhlke et al., 1994; Wester et al., 1996). The procedure represents an alternative approach to the production of  $N$ -succinimidyl 3- and 4-radioiodobenzoate accomplished via iododestannylation which evolves the laborious synthesis of the tin precursor (Zalutsky and Narula, 1987; Wilbur et al., 1989). The reaction conditions were optimized and a radiochemical purity of more than 90% was obtained when using  $160^\circ\text{C}$ , 60 min reaction time and a [CuCl]/ $p$ -bromobenzoic acid] relation of about  $10^{-2}$ . Moerlein (1990) also observed the same relation to the iodobromination of bromobenzene, but with radiochemical yield not greater than 60%. Despite obtaining good radiochemical yields at  $155^\circ\text{C}$  and 60 min, the radiochemical yield using the same temperature and 30 min was only  $38.73 \pm 2.61$  ( $N = 3$ ). The influence of time is specially important when considering labeling conditions with iodine radioisotope with short physical half life, such as iodine-123. In our studies, the HPLC analysis of labeling mixtures using different [CuCl]/ $p$ -bromobenzoic acid] relation, in a C18 column ( $10 \mu\text{m}$ ,  $4.6 \times 250 \text{mm}^2$ ) and methanol:distilled water gradient revealed that, using [CuCl]/ $p$ -bromobenzoic acid] relation of about  $10^{-3}$ , a third peak was observed in the radioactive HPLC chromatogram, with a  $R_t$  between the  $R_t$  of the  $p$ - $^{131}\text{I}$ iodobenzoic acid and of the  $^{131}\text{I}$ iodine with intensity proportional to the CuCl concentration. This radiochemical species is also identified in the paper chromatographic system used and referred to as “another impurity”.

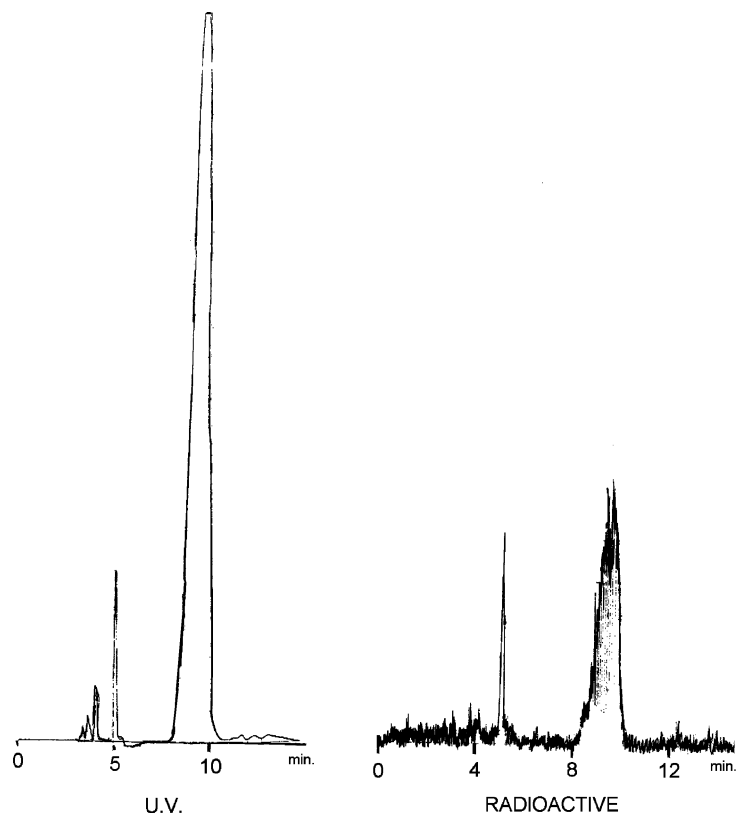


Fig. 3. HPLC profile of radioiodinated prosthetic group without Sep-Pack purification; silica column,  $10\ \mu\text{m}$ ,  $4.6 \times 250\ \text{mm}^2$ ; hexane:ethylacetate:acetic acid (70:30:0.2); flux: 1 ml/min.

The methodology employed in the purification of the *p*-radioiodo benzoic acid using a C-18 Sep-pak was effective to separate the free iodine, but was not able to purify the radioiodinated 4-iodobenzoic acid from the precursor 4-bromobenzoic acid. The UV HPLC spectrum of the purified fraction exhibits a peak with  $R_t = 5.15\ \text{min}$ , that corresponds to the bromoprecursor (Fig. 2).

The *p*- $^{131}\text{I}$ iodobenzoic acid reacted with TSTU to produce the *N*-succinimidyl 4- $^{131}\text{I}$ iodobenzoate in a radiochemical yield greater than 98%. The concentration of the NaOH employed and the absence of water in the reaction medium were determinant factors to obtain high radiochemical yields. However, the protein conjugation using the *N*-succinimidyl 4- $^{131}\text{I}$ iodobenzoate resulted in a low radiochemical yield. Antibodies and other proteins have been radioiodinated via the addition of both *p*- (Wilbur et al., 1989) and *m*-substituted (Zalutsky and Narula, 1987) iodophenyl groups via the corresponding-*N*-succinimidyl (tri-*n*-butylstannyl)benzoate, with different radiochemical yields varying from 40% to 90% in the first case and from 33% to 60% in the second case, using Sep-Pack or HPLC purified active esters.

In our studies, despite the alteration of labeling parameters as protein concentration, incubation time and solvent reaction mixture, the radiochemical yield did not increase significantly. The presence of bromoprecursor in the reaction medium may explain, in part, the poor radiochemical yield when considering the coupling of the radioiodinated prosthetic group to the protein. In fact, the presence of the bromoprecursor in the reaction medium is more directly related to the final specific activity of the labeled protein than to the radiochemical yield. Another fact that contributed to the low incorporation of the radioiodinated prosthetic group to the protein was the incorporation of part of the radioactivity in the reaction vial (glass or polystyrene). Additional studies, not described in this work, were conducted on labeling the peptide Octreotide (Sandoz) using the *N*-succinimidyl 4- $^{131}\text{I}$ iodobenzoate produced. Radiochemical yield observed in this case was superior than 87%, with minimal radioactivity retention in the reaction vial. It seems that the prosthetic group produced by the alternative way is more effective on labeling peptides than antibodies.

Despite obtaining low radiochemical yields on the labeling IgG with the prosthetic group, biodistribution

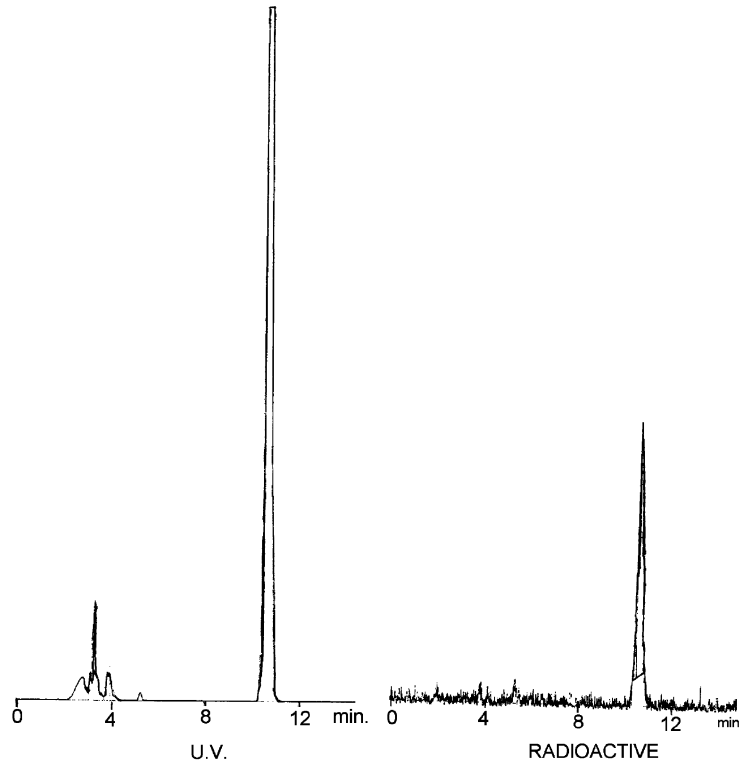


Fig. 4. HPLC profile of radioiodinated prosthetic group after Sep-Pack purification; silica column, 10  $\mu\text{m}$ , 4.6  $\times$  250 mm<sup>2</sup>; hexane:ethylacetate:acetic acid (70:30:0.2); flux: 1 ml/min.

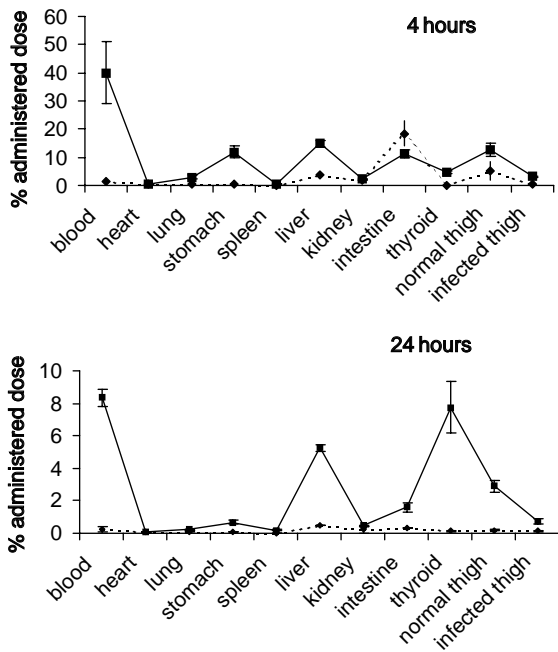


Fig. 5. Percent administered dose/organ at 4 and 24 h after administration of [<sup>131</sup>I]IgG labeled by direct (—) and indirect (---) labeling protein approaches in tupertine-promoted inflammation focus animals.

studies were performed in order to compare the in vivo stability of the protein labeled by direct and indirect methods. Biodistribution studies showed that the animal group that received the IgG labeled by the radioiodinated prosthetic group presented fast blood clearance and low thyroid and stomach uptake when compared to the group that received the IgG labeled by Iodogen method ( $P < 0.01$ ). The reduced thyroid and stomach uptake indicated that the protein labeled by the prosthetic group is less susceptible to the enzymatic in vivo dehalogenation process. The uptake on the infected thigh is numerically higher when using direct labeled IgG. However, when comparing the infected thigh/blood relation, it is of 3.73 to the group administered with IgG labeled with prosthetic group and 0.31 to the group administered with IgG labeled by direct method, 4 h after the dose administration. This result suggests that the indirect labeling method results in a biological distribution pattern more compatible with early imaging acquisition.

### 5. Conclusions

The synthetic procedure presented represents a good alternative for the production of a radioiodinated prosthetic group with good radiochemical yield for

labeling proteins with in vivo stability. This method is a good alternative to the iododemetalation methods for preparing SIB in specific cases where high specific activity conjugated products are not required. The potential of this technique may be further investigated for labeling peptides, of clinical interest for the use with radioiodine.

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