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RESEARCH PAPER

Comparison of Succinimidyl [^{125}I]Iodobenzoate with Iodogen Iodination Methods to Study Pharmacokinetics and ADME of Biotherapeutics

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ABSTRACT

Purpose To assess the application of succinimidyl iodobenzoate (SIB) iodination method in labeling biotherapeutics to study their pharmacokinetics (PK) and biodistribution.

Method An IgG molecule (protein-01) and a 40 KDa protein (protein-02) were evaluated. Pharmacokinetics (PK) and biodistribution of the radiolabeled IgG (^{125}I-protein-01) in mice compared parameters from SIB and Iodogen protein iodination labeling methods. In addition, PK of radiolabeled 40 KDa protein (^{125}I-protein-02) using SIB was compared with non-labeled protein-02 analyzed by ligand binding assay (LBA).

Results Up to 72 h following a single IP injection to mice, the percentage of "free-label" determined by the soluble counts after TCA precipitation to total radioactivity in serum samples was 2.8–49.4% *vs.* <1.0% for ^{125}I-protein-01 iodinated *via* Iodogen or SIB methods, respectively, suggesting a higher *in vivo* stability of ^{125}I-protein-01 labeled *via* the SIB method. The serum exposure of ^{125}I-protein-01 was two-fold higher, and correspondingly, the tissue exposure was also higher (averaging 3.6 fold at 168 h) when using SIB protein labeling method than when using the Iodogen method. In addition, following subcutaneous (SC) administration to mice, the serum exposure of ^{125}I-protein-02 labeled *via* SIB method was similar to protein-02 measure by LBA.

Conclusion In this study, iodination of proteins using SIB methodology has overcome the dehalogenation problem *in vivo* which is inherent in Iodogen method, and PK parameters of a protein iodinated *via* SIB were comparable to the un-labeled protein measured by LBA. The SIB iodination method is an improved labeling approach for biotherapeutics used in studying PK and biodistribution characteristics.

KEY WORDS ADME · biotherapeutics · pharmacokinetics · radioiodination · succinimidyl [^{125}I]iodobenzoate

ABBREVIATIONS

Ab	Antibody
ADME	Absorption, distribution, metabolism, excretion
AUC	Area under concentration *versus* time curve
IP	Intraperitoneal
LBA	Ligand-binding assays
LC-MS	Liquid chromatography-mass spectrometry
mAb	Monoclonal antibody
MW	Molecular weight
NCS	N-chlorosuccinimide
PBS	Phosphate buffered saline
PK	Pharmacokinetics
RCP	Radiochemical purity
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatogram
SIB	Succinimidyl iodobenzoate
TCA	Trichloroacetic acid

INTRODUCTION

It is well recognized that, for many monoclonal antibodies (mAbs) and related drug modalities, such as Fc-fusion proteins, bispecific mAbs and recombinant proteins, that the pharmacological target is often located within the tissue. Thus, making quantification of biotherapeutic tissue concentrations an

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important determinant of their pharmacodynamic (PD) and, sometimes, exaggerated-PD/toxic effects. It is therefore important to characterize and accurately predict the biodistribution of mAbs to better understand the dose–response relationship (1).

Conventional analytical assays for measuring the concentration of biotherapeutics in plasma or serum rely on ligand binding assays (LBA) (1,2). Advantages of LBA include high specificity and sensitivity, ease of sample handling and relatively low cost. However, unless reagents are already available, such as when using generic anti-Fc reagents or commercially available kits, developing specific reagents for LBA designed to measure biotherapeutics is time consuming and a labor intensive process (3–5). Another approach for quantitative measurement of biotherapeutics in plasma or serum and tissue homogenates is mass spectrometry (6). Advantages of LC-MS/MS over LBA include improved selectivity between structurally or chemically similar peptides and proteins and potentially no requirement to develop specific reagents (7). To further improve the sensitivity and reduce the interference of peptide fragments originating from endogenous proteins, immune-absorption is usually applied (requiring the use of anti-peptide antibodies or commercially available specific reagents) in the sample extracting process prior to LC-MS/MS, which is particularly important for tissue homogenates or plasma samples with low drug concentrations (6). Both LBA and LC-MS/MS require method development to accurately quantify protein concentrations which may be time consuming depending on the difficulties encountered, *e.g.*, matrix interferences, sensitivity requirements *etc.*

Alternatively, a radioisotope probe assay that directly measures radioactivity of a radioisotope conjugated to the biotherapeutic provides a faster and more economical initial approach to assess PK and biodistribution than LBA or LC-MS/MS methods which require reagent assessment and assay development time. The radioisotope labeled molecule is then quantitatively analyzed based on its radioactive emission. Owing to the high sensitivity, quantitative method, minimal sample matrix interference, ease of sample handling (*e.g.*, direct measurement of gamma radioactivity of ^{125}I-labeled protein in tissue samples without the need for extractions or homogenization), and favorable cost, this approach has an important place in early preclinical drug research and development of biotherapeutics (8–10).

The radioisotopes most commonly used in labeling of biotherapeutics for preclinical PK/ADME studies include ^{18}F, ^{99m}Tc, ^{32}P, ^{14}C, ^{35}S,^{111}In, ^{131}I, ^{123}I and ^{125}I (10). Among this list, ^{125}I is often the first choice for biotherapeutics owing to its low cost, well known and established iodination chemistry for proteins, ideal physical properties of gamma emission (35 keV) and 60-day decay half-life (9,11–14).

The chemistry used to radioiodinate proteins has been studied extensively (15,16); early methods are based on direct-radioiodination using Na^{125}I in the presence of oxidants, such as chloramines-T (17) or tetrachlorodiphenylglycouril (Iodogen) (18), which involves substitution of the iodine ortho to the hydroxyl groups of tyrosine residues in the protein. However, in addition to the potential impact on bioactivity of proteins being directly exposed to oxidizing reagents during the iodination (18), the radiolabeled proteins formed by these methods as well as using Bolton-Hunter reagent (N-succinimidyl para-hydroxyphenylpropionate) (19) may undergo various degrees of *in vivo* enzymatic deiodination due to their structural similarities to thyroid hormone (20) and the weakening of carbon-iodine bond by the ortho-hydroxyl group substitution (13). One approach to evaluate the degree of *in vivo* deiodination and obtain a more accurate assessment of intact protein concentration is to use Trichloroacetic acid (TCA) to precipitate protein and assume that the TCA precipitable counts represent the majority of intact proteins and %free from the precipitation represents the degree of deiodination and protein degradation.

Historical data from our lab have demonstrated that IgG MAbs in general behave well in terms of degradation and clearance. Systemic PK parameters agree well from either ^{125}I labeled or LBA measured proteins with very few exceptions. The %free from TCA precipitation of tissue homogenate ranges from 2% to 10% across a panel of tissues including liver, kidney, lung and spleen (unpublished data), thus routinely we use total tissue counts to calculate IgG tissue concentrations. However, acute de-iodination can occur in our radioiodinated (Iodogen) antibody fragments and small recombinant proteins in addition to their own *in vivo* degradation, which would lead to over- or under-estimation of PK parameters. Sometimes, follow up analysis will be conducted to confirm the integrity of labeled protein including TCA precipitation of tissue homogenate (21), HPLC or SDS-PAGE followed with autoradiography.

In an attempt to overcome the *in vivo* instability of radiolabeled proteins, some PK/ADME studies utilize heavy metal radioisotopes, such as ^{111}In, or residualizing radioiodine labeling. The use of heavy metal radioisotopes or residualizing radioiodine labeling have been extremely helpful in understanding the internalization of biotherapeutics (an uptake process after binding to the membrane-bound target), because these labels are resistant to lysosomal degradation and remain within the cell for extended periods of time post release from the biotherapeutic (22,23). Although heavy metal radioisotopes and residualizing iodine labeling are successfully used in tumor radioimmunodetection and/or radioimmunotherapy, they are not ideal choices for biotherapeutic PK/ADME studies, as the concentration measurements of intact biotherapeutics in the blood circulation and tissues is the main objective of these types of investigations. The N-succinimidyl [^{125}I]iodobenzoate (SIB) iodination of proteins is an indirect-iodination method conjugating SIB, as an intermediate, onto amine groups of lysine

residues and has several advantages: a) eliminates the harsh oxidizing conditions during labeling, b) minimizes the potential of *in vivo* deiodination occurring compared to direct-radioioinated proteins (12,16,24,25) and c) any metabolized SIB-lysine fragment will be readily excreted through the kidney into the urine (26). In this investigation, PK and biodistribution of a radiolabeled IgG (^{125}I-protein-01) in mice were compared using either SIB or Iodogen protein labeling methods. In addition, PK of a radiolabeled protein with molecular weight of 40 KDa (^{125}I-protein-02) was evaluated and compared with un-labeled protein-02 analyzed by LBA. In this manuscript, to facilitate reading, proteins (protein-01 and protein-02) that are labeled *via* SIB or Iodogen method will be named protein(-01 or -02)-SIB or protein(-01 or -02)-Iodogen; protein-02 that was dosed without labeling and whose serum concentrations were analyzed by LBA will be named protein-02-LBA.

MATERIALS AND METHODS

N-Succinimidyl-3-(tri-n-butylstannyl) benzoate ($C_{23}H_{35}NO_4Sn$; MW = 508.23) was synthesized by Texas Biochemicals Inc. Iodogen (Pierce Iodination Reagent) was purchased from Thermo Scientific. N-chlorosuccinimide (NCS), sodium bisulfate ($NaHSO_3$), methanol, acetic acid, and sodium ascorbate were purchased from Sigma-Aldrich. Iodine-125, 100 mCi/mL in 0.01 mM sodium hydroxyl (NaOH), was bought from Perkin Elmer. PD-10 column and Sep-Pak silica gel column were obtained from GE Healthcare. Protein-01 and -02 were supplied by Pfizer Inc.

Protein Radioiodination, Purification, Characterization and Dosing Solution Preparation

The application of SIB labeling method was demonstrated in PK studies using two proteins: protein-01 and protein-02 where protein-01 is a monoclonal antibody with a molecular weight of approximately 200 KDa, and protein-02 is a 40 KDa protein. ^{125}I-labeling of mAb protein-01 (^{125}I-protein-01) was conducted using either Iodogen or SIB iodination method. All the reactions were performed inside the iodination hood in compliance with radiation safety requirements. In general, for the Iodogen iodination method, an aliquot of Na^{125}I (10 μL, ~1.2 mCi), 0.1 mg of the protein test article in phosphate buffered saline (PBS) (with an approximately 1:1 M ratio for ^{125}I and the antibody), together with 100 μL of 0.1 M, pH 7.2 phosphate buffer were added into a 10 μg Iodogen pre-coated reaction vial. After sealing the tube with a cap, the reaction solution was immediately mixed *via* gently mixing for 30 s. The reaction mixture was then kept at ambient temperature for 10 min, with a 10-s swirling at minute intervals. Then the reaction was quenched by adding 0.2 mL of 20 mg/mL sodium ascorbate. The ^{125}I-labeled product was purified from non-reacted free ^{125}I remaining in the quenched reaction solution using a pre-buffered PD-10 column (non-specific protein binding sites in the column were blocked with bovine serum albumin, the column then washed with at least three column volumes of PBS).

For SIB iodination method, briefly, an aliquot of Iodine-125 (10 μL, ~ 1.2 mCi) was reacted with 2.5 μg N-Succinimidyl-3-(tri-n-butylstannyl) benzoate together with 10 μg NCS as an oxidant in 50 μL of methanol containing 1.5% acetic acid (v:v). After 15 min incubation at ambient temperature, the remaining oxidant in reaction solution was quenched by adding 10 μg sodium bisulfate (reducing agent) and incubating at ambient temperature for an additional 5 min. The ^{125}I-labeled intermediate (SIB) was purified from free ^{125}I and other chemicals remaining in the reaction solution using a Sep-Pak silica gel column. The ^{125}I-labeled SIB was conjugated to protein test articles (with an approximately 1:1 M ratio of intermediate to protein test article) from the incubation at ambient temperature for 20 min at pH 8.0. The radiolabeled product was purified using a pre-balanced PD-10 column, eluted in PBS. A scheme of the iodination procedure is shown in Fig. 1.

The radiochemical purity (RCP) of ^{125}I-protein-01 product was evaluated *via* size exclusion chromatogram (SEC) equipped with an in-line radioactive detector. For SEC analysis, approximately 1 μCi of ^{125}I-protein-01 product solution was injected into the HPLC (Agilent 1100) equipped with a size exclusion column (Agilent SEC-5, 150 A, 7.8 × 300 mm) and eluted with a flow rate of 1 mL per minute and mobile phase of 25 mM phosphate buffer, 0.15 M NaCl, pH 6.8. The identity of ^{125}I-protein-01 was confirmed by comparing its retention time from radiometric chromatogram to that of the reference protein in UV (280 nm) chromatogram.

The dosing solution was prepared by mixing ^{125}I-protein with unlabeled protein (protein stock solution), and formulation buffer (PBS, pH 7.2) to achieve the desired final protein concentration. The dosing solution concentrations for protein-01 and protein-02 were 1.6 mg/mL and 1.25 mg/mL, with dosing volume of 5 mL/kg and 4 mL/kg to reach dose level of 8 mg/kg and 5 mg/kg in mice, respectively. The radioactive specific activity of the final dosing solutions was approximately 35 μCi/mg and 58 μCi/mg, respectively. The RCP of the final dosing solution was also assessed by using trichloroacetic acid (TCA)-precipitation measurement of the percentage of free iodine. Briefly, aliquots (5 μL, in triplicates) of the dosing solution were mixed with mouse serum (195 μL) and the mixtures were counted for total counts (cpm, counts per minute) with a gamma counter (Model 2470 PerkinElmer). An equal volume of 20% TCA solution (200 μL of 20% TCA stock solution) was added and mixed with the sample. The sample mixture was centrifuged at 10,000 g for 5 min to precipitate the protein.

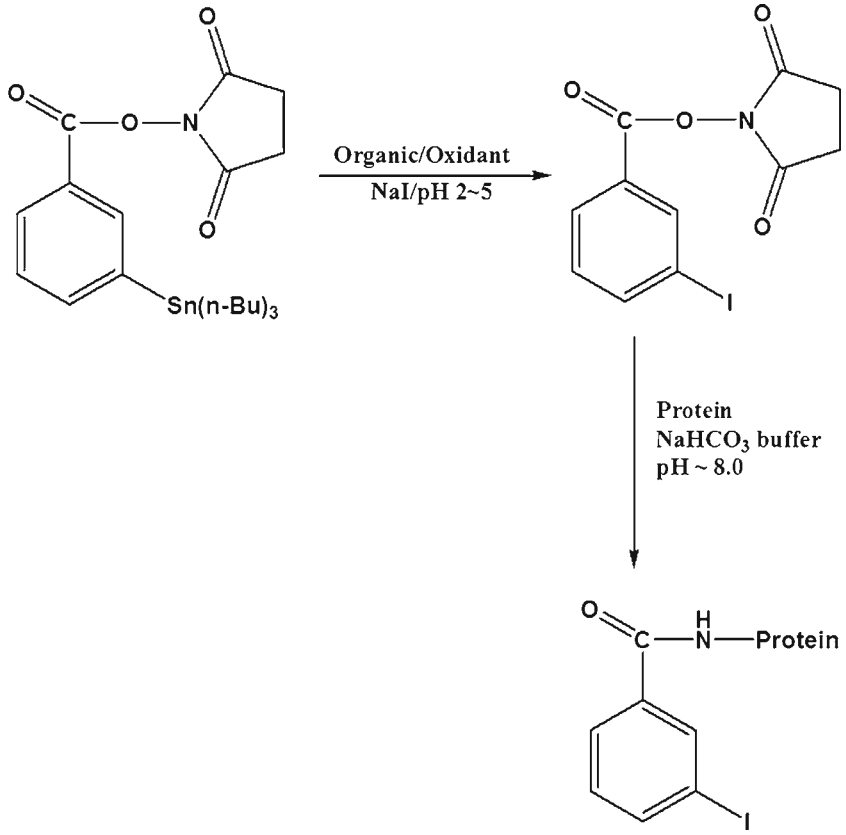

Fig. 1 A schematic of SIB protein iodination method.

The radioactivity in resultant supernatant was measured; the %free based on soluble radioactive counts (cpm) was calculated. An RCP of >95% was required for the final dosing solution. The specific activity of the final dosing solution (μCi/mg) was calculated by the following formula: (TCA precipitable cpm)/[dosing solution concentration (mg/mL)* aliquot dosing solution volume (0.005 mL)*2,200,000 (cpm/μCi)]. No counting efficacy correction was applied in the calculation of specific activity of the dosing solution since a similar counting geometry was used for all sample measurements (*i.e.* dosing solution standards, serum samples and tissues of interest).

Animal Studies

For the animal studies, mice (~8 week old) with a body weight range of 20–30 g were purchased from Charles River Lab (Wilmington, MA) and Pfizer Institutional Animal Care and Use Committee approved all aspects of these studies. All studies were performed in accordance with the National Institutes of Health guide for the care and use of animal resources. The mice were pretreated with KI water (20 mM) approximately 3 days prior to the dosing to minimize thyroid uptake and to increase the whole body clearance for any potentially unbound free ^{125}I generated *in vivo*. 8 mg/kg of ^{125}I-protein-01-Iodogen or ^{125}I-protein-01-SIB was given *via* intraperitoneal (IP) route and 5 mg/kg of ^{125}I-protein-02-SIB was given *via* subcutaneous (SC) route. Non-radiolabeled protein-02-LBA was also given at a 5 mg/kg dose *via* SC administration. At predetermined time-points post dosing ($n=3$ per time point), blood samples were separately collected either by cardiac puncture or retro-orbital bleeding into serum separator tubes. Serum samples were harvested by centrifugation of the blood samples at 3,000 g for 5 min.

For tissue harvesting, at predetermined time-points ($n=3$ per time point), animals were sacrificed after blood collection and tissues of interest were collected after a whole body vascular perfusion to remove the blood content. Whole body vascular perfusion was conducted by administering at least 20 mL of heparin-PBS (25 units per mL) for approximately 10 min. The tissues of interest including brain, mesenteric lymph nodes, skin, visceral fat, skeletal thigh muscle, lung, heart, spleen, liver, stomach, small intestine, large intestine, and kidney were collected and weighed. The contents in stomach, small intestine and large intestine were not removed. The radioactivity (total counts in cpm) of the tissue samples was measured by the gamma counter.

Determination of Radioactive Equivalent Concentrations in Serum and Tissues

The serum radioactive equivalent concentrations (ng eq./mL) of ^{125}I-protein were determined based on the measured TCA precipitable (protein associated) radioactivity. For TCA precipitation, the aliquot (50 μL) of serum sample was mixed with 150 μL of normal mouse serum, followed by the addition of 200 μL of 20% TCA solution. The sample mixture was spun at 10,000 g for 5 min to precipitate the protein. Total and TCA-soluble radioactivity in the supernatant was determined. TCA-precipitable radioactivity (cpm) in a given sample, the specific activity of the dosing solution (TCA-precipitable cpm per mg of protein), as well as dates of sample (T_S) and dosing solution (T_D) measurements, were used to calculate the equivalent concentration of test article (ng eq./mL) in a given sample, using the formula:

$$\frac{TCA - precipitable\ counts}{e^{-\left(\frac{0.693}{60.2}\times(\mathrm{Ts}-\mathrm{Td})\right)} * (specific\ activity * sample\ volume)}$$

The radioactive equivalent concentration (ng eq./g) of ^{125}I-protein-01 in tissues was calculated based on the measured total radioactivity in tissue samples and the specific activity of the dosing solution after a correction for physical decay half-life of ^{125}I, using the formula:

$$\frac{Sample\ counts}{e^{-\left(\frac{0.693}{60.2}\times(\mathrm{Ts}-\mathrm{Td})\right)} * (specific\ activity * sample\ weight)}$$

No homogenization and TCA-precipitation were performed for tissue samples.

LBA Methods for protein-02 on Meso Scale Discovery (MSD)

A protein-02 specific capture antibody (Pfizer, Inc.) was diluted in phosphate buffered saline (pH 7.2) at the appropriate concentrations and incubated overnight at 4°C, 100 μL/well using 96-well Multi Array plates (Meso Scale Discovery, Rockville, MD). Plates were washed three times with wash buffer (50 mM Tris, 1 M NaCl, 0.05% Tween-20), and unbound sites blocked with 150 μL/well block buffer for 1–2 h (phosphate buffered saline, 0.05% Tween-20, 4% (w/v) bovine serum albumin (BSA), 0.05% Proclin300). After blocking, plates were washed three times with wash buffer. Protein-02 top standard point was prepared at 40× concentration in neat pooled mouse serum (Bioreclamation, Inc., Westbury, NY) and then diluted to the minimum required dilution (MRD) of 1:40 in assay buffer (PBST, 1% (w/v) BSA, 0.05% (v/v) Proclin 300). Subsequent standard curve points were made with a two-fold dilution series in assay buffer containing 2.5% pooled mouse serum (assay diluent) for a total of twelve standard points ranging from 800 to 0.391 ng/mL in 2.5% serum. The assay diluent served as the blank. Controls were prepared in neat serum at levels covering the range of quantitation and subsequently diluted to the MRD in assay buffer. Based on estimated sample concentrations, samples were diluted to fall within the range of quantitation. All samples were diluted 1:40 in assay buffer with subsequent dilutions, if necessary, in assay diluent containing 2.5% pooled mouse serum. Standards, controls and test samples were dispensed to the assay plates at 100 μL/well and incubated for 1.5 h with shaking at room temperature, followed by three washes with wash buffer. Ruthenylated anti-protein-01 detector antibody was diluted in assay buffer, dispensed at 100 μL/well, and incubated for 1 h with shaking at room temperature. Following three washes with wash buffer, Read Buffer solution (Meso Scale Discovery, Rockville, MD) was added at 150 μL/well. Electrochemiluminescent signal was read on the Sector Imager 6000 MSD reader (Meso Scale Discovery, Rockville, MD) and sample concentrations were determined by interpolation from a standard curve that was fit using a 4-parameter logistic equation with $1/Y^2$ weighting.

Pharmacokinetic Calculations

PK parameters were calculated with the mean serum or tissue concentrations of three mice at each time point. A non-compartmental analysis module (Model 200) of the pharmacokinetic software package WinNonlin (version 5.1, Pharsight) was used. The area under the concentration *versus* time curve (AUC) was calculated using the linear trapezoidal method. The slope of the apparent terminal phase was estimated by log-linear regression using at least three data points and the terminal rate constant (λ) was derived from the slope. $AUC_{0-\infty}$ was estimated as the sum of the AUC_{0-t} (where t is the time of the last measurable concentration) and C_t/λ. The apparent terminal half-life ($t_{1/2}$) was calculated as $0.693/\lambda$.

Characterization of Serum Sample *via* SDS-PAGE/Autoradiography

The serum sample of ^{125}I-protein-01 was characterized by non-reducing electrophoresis (Invitrogen). Briefly, a serum sample was mixed with 2× sample buffer. The mixture was heated at 70°C for 10 min, and then loaded onto 10% SDS-PAGE gel. The dosing solution of ^{125}I-protein-01 treated in the same manner was used as a control. Following electrophoresis, the gel was impregnated with gel dry solution (30% methanol and 5% glycerol in water) for 15 ~ 20 min at ambient temperature, covered in Saran wrap and dried at 80°C for 1.5 h. For autoradiography, the gel was exposed to a phospho screen (Molecular Dynamics) and then imaged (Storm 860).

Statistical Calculation

The statistical analysis was performed using the Student's *t*-test for unpaired data between two different methods.

RESULTS

Protein Radioiodination and Dosing Solution Characterization

A labeling yield of approximately 80% or 40% was obtained for ^{125}I-labled mAb *via* Iodogen or SIB method, respectively. Compared to the Iodogen method, the yield using SIB method is relatively low because of potential hydrolysis of N-succinimidyl coupling moiety from the intermediate in aqueous buffer (11,16). An RCP of greater than 98% with <2% free ^{125}I was obtained for purified ^{125}I-protein-01 product and no other impurities, *e.g.*, aggregates, were observed for ^{125}I-protein-01 product prepared *via* either iodination methods (Iodogen or SIB). A representative example of SEC analysis of ^{125}I-protein-01-SIB is shown in Fig. 2.

Serum Equivalent Concentrations, Pharmacokinetics, and Characterizations

The radioactive equivalent mouse serum concentration profiles following a single IP dosing of ^{125}I-protein-01 prepared by both iodination methods at 8 mg/kg are shown in Fig. 3a. The serum concentration profile measured by TCA-precipitable radioactivity or LBA following SC dosing of 5 mg/kg of ^{125}I-protein-02-SIB or protein-02-LBA are shown in Fig. 3b. Sample counts in cpm of less than three times of background (approximately 30 cpm) was set as the limit of quantitation (LOQ), and was treated as zero for the calculation of mean and standard deviation (SD). For protein-01, a comparable equivalent concentration (ng eq./mL) was observed at 1-h post injection for ^{125}I-protein-01 prepared by either iodination methods, but the concentration of ^{125}I-protein-01-Iodogen dropped much more rapidly comparing to ^{125}I-protein-01-SIB (Fig. 3a). The concentration ratio of ^{125}I-protein-01-Iodogen to ^{125}I-protein-01-SIB decreased from 91% at 1 h to 22% at 72 h post dosing (Table I). Protein concentrations in the serum samples collected at 240, and 336 h post injection of ^{125}I-protein-01-Iodogen were less than LOQ (data not shown). For protein-02, the serum concentrations of ^{125}I-protein-02-SIB and protein-02-LBA were comparable and within ±30% assay acceptability criteria used in discovery research (Fig. 3b).

Table II summarizes mouse PK parameters of ^{125}I-protein-01 prepared by both iodination methods and those of ^{125}I-protein-02-SIB and protein-02-LBA. Following a single 8 mg/kg IP dose, a drug exposure for ^{125}I-protein-01-SIB was approximately two times of that for ^{125}I-protein-01-Iodogen. The lower AUC and shorter half-life of ^{125}I-protein-01-Iodogen are consistent with the sharper decrease of the serum concentrations at later time points. The $AUC_{0-\infty}$

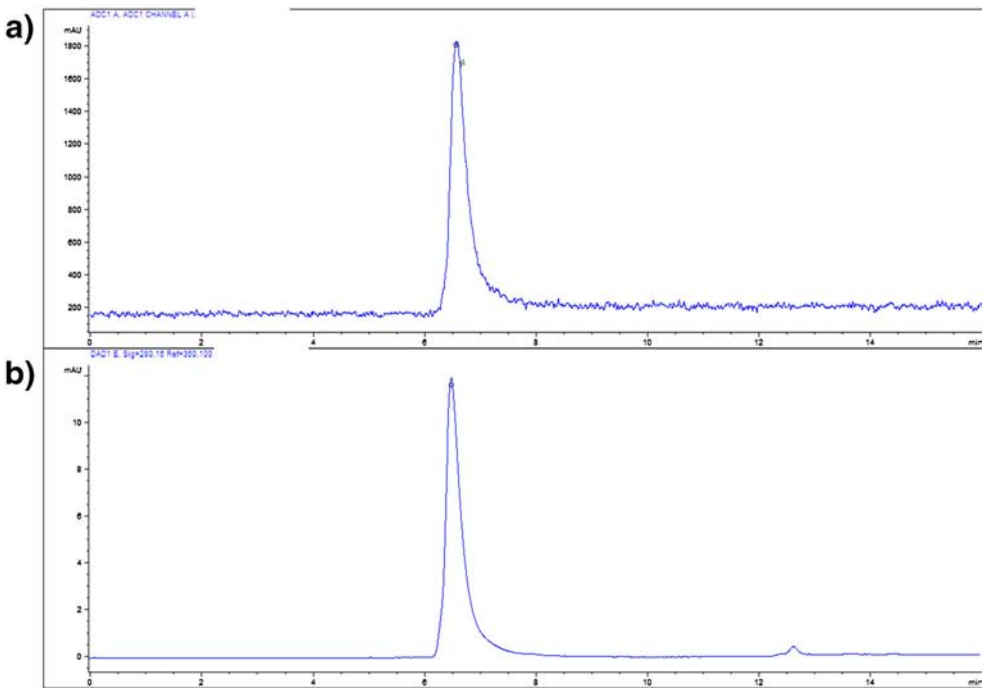

Fig. 2 SEC characterization of ^{125}I-protein-01 labeled *via* SIB iodination method. Radiometric analysis of ^{125}I-protein-01-SIB (**a**), chromatogram of unlabeled protein-01, 3 μg, UV $\lambda = 280$ nm (**b**).

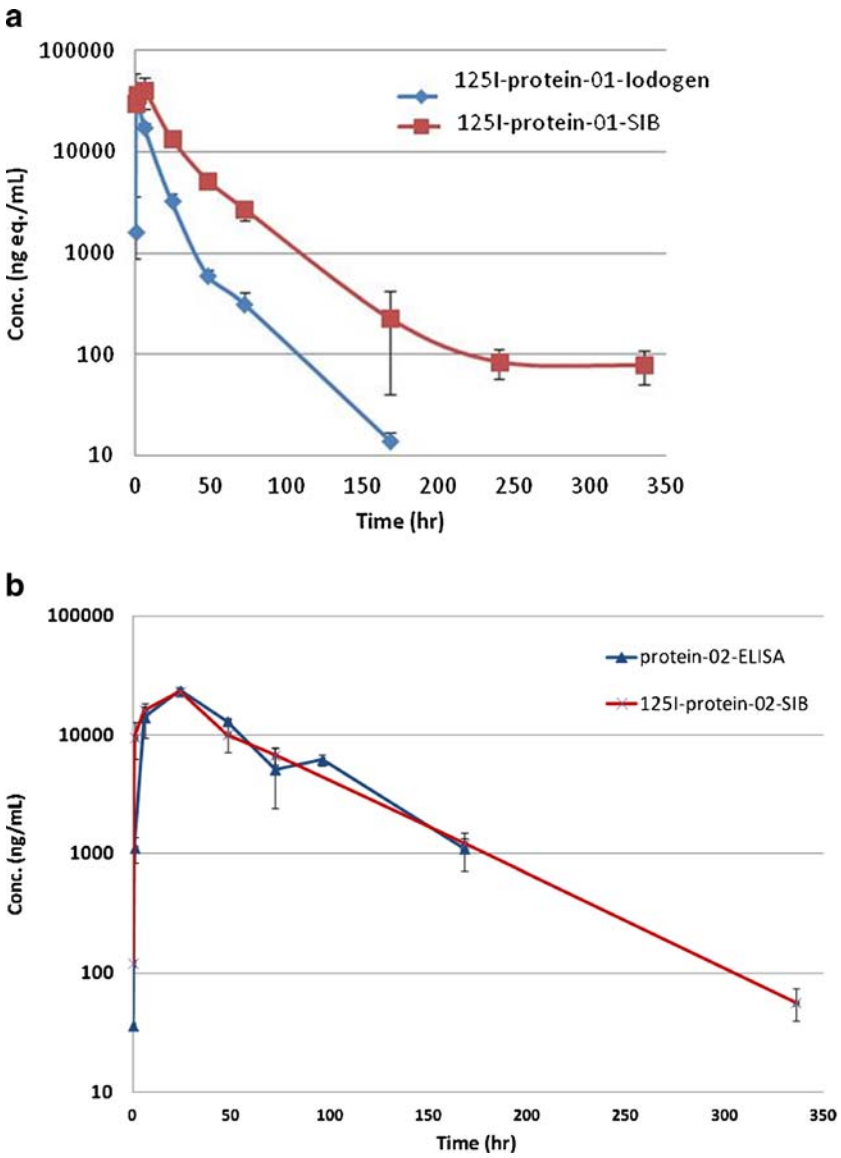

Fig. 3 Mean (±SD) mouse serum equivalent concentration (ng eq./mL) following *in vivo* dosing. Profiles following (**a**) a single IP dose of 8 mg/kg of ^{125}I-protein-01 prepared *via* SIB or Iodogen iodination method, or (**b**) a single SC dose of 5 mg/kg of ^{125}I-protein-02-SIB or protein-02-ELISA ($n = 3$ per time point). A value of <LOQ was observed at 240 and 336 h time points for ^{125}I-protein-01-Iodogen.

for both ^{125}I-protein-02-SIB and protein-02-LBA following a single 5 mg/kg SC dose were comparable.

The radioactivity present in the supernatant after the TCA precipitation, referred to as "free label", represents free ^{125}I and/or radioactive small protein fragments proteolysed from intact proteins that could not be precipitated due to their small molecule size. The percentage (%) of radioactivity in the TCA supernatant, considered %free counts, for the dosing solutions and serum samples is summarized in Table III. The %free counts are believed to be mainly composed of free iodine due to de-halogenation that cannot be precipitated by TCA. The %free counts measured at the time of dosing solution preparation was 2% following Iodogen method and 0.1–0.5% following SIB method. Following IP administration, a significant %free counts in serum was present for ^{125}I-protein-01-Iodogen (ranging from 2.8 to 49%), but was found to be low (<1% at all measureable time points) for serum samples following IP administration of ^{125}I-protein-01-SIB (Table III). Following SC administration of ^{125}I-protein-02-SIB, a low %free counts was also observed in serum samples up to 72 h except for 5 min at 27%, and the

Table I TCA-Precipitable Serum Equivalent Concentration Ratio between ^{125}I-protein-01-Iodogen and ^{125}I-protein-01-SIB, Following a Single IP Dose of 8 mg/kg to Mice (Mean ± SD; $n = 3$ per Time Point)

	Time post IP injection				
	1 h	6 h	24 h	72 h	168 h
Serum concentration ratio ^{125}I-protein-01-Iodogen/^{125}I-protein-01-SIB	91%	44%	24%	22%	ND

ND not determined

Table II PK Parameters of ^{125}I-protein-01, Prepared *via* SIB or Iodogen Iodination Method, Following a Single 8 mg/kg IP Dose to Mice, and ^{125}I-protein-02-SIB and protein-02-LBA Following a Single 5 mg/kg SC Dose to Mice

Iodination method	C_{max} (μg eq./mL)	T_{max} (h)	$AUC_{0-\infty}$ (h*μg eq./mL)	$t_{1/2}$ (h)
^{125}I-protein-01-SIB	40.0	6	1,134	24.8
^{125}I-protein-01-Iodogen	33.1	1	538	18.7
^{125}I-protein-02-SIB	23.1	24	1,502	38.5
protein-02-LBA	23.6	24	1,440	29.0

PK parameters were calculated based on mean TCA-precipitable radioactive equivalent serum concentrations, determined by gamma-counter for ^{125}I-protein-01-SIB, ^{125}I-protein-01-Iodogen, ^{125}I-protein-02-SIB; and ligand binding assay for protein-02-LBA. Individual concentration values <LOQ (defined as 3× background cpm) were treated as zero for calculations of the mean and SD; $n = 3$ per time point

percentage started to increase to 9.2% at 168 h and 54% at 336 h.

Mouse serum samples collected from different time points up to 72 h post injection of ^{125}I-protein-01-SIB were further analyzed by non-reducing SDS-PAGE followed by autoradiography. The results showed that the intact drug (~200 kDa) band and a lower MW (~110 kDa) band were present in both the formulated biotherapeutic dosing solution and serum samples (Fig. 4). The observed lower MW band (~110 kDa, monomer form of protein-01) was in agreement with the profile of unlabeled IgG protein-01 analyzed by SDS-PAGE in the same manner (data not shown). Except for the intact drug and monomer bands, no other aggregated or smaller protein fragment bands were observed in serum samples collected up to 72 h post IP injection, which is consistent with the low %free counts. The results illustrated that the measured serum equivalent concentrations (ng eq./mL) for ^{125}I-protein-01-SIB could fully represent the drug concentration of the protein-01 used in this investigation.

Tissue Distribution and Tissue/Serum Ratios

The total radioactive equivalent concentrations (ng eq./g) in tissues of interest as well as serum (ng eq./mL) for ^{125}I-protein-01 prepared *via* SIB or Iodogen are shown in Fig. 5a and b, respectively, following a single 8 mg/kg IP dose to mice. A parallel concentration profile relative to serum was observed for all tissues tested at early time points, up to 168 h (Fig. 5), followed by a slower decline of the radioactivity in tissues at later time points. Compared to other tissues, the radioactive equivalent concentration in brain was very low, suggesting minimum penetration of ^{125}I-protein-01 through the blood–brain barrier.

For further comparison, statistical analysis was conducted comparing the tissue concentrations between ^{125}I-protein-01-SIB and ^{125}I-protein-01-Iodogen as illustrated in Fig. 5c and d. Except for stomach, there was no significant difference in concentrations in tissues at 1-h time point for ^{125}I-protein-01 prepared by either iodination methods (Iodogen *vs.* SIB). However, the radioactive equivalent concentration was significantly lower in several tissues at later time points for ^{125}I-protein-01-Iodogen, which is consistent with the faster serum concentration decline (Fig. 5d). Table IV lists the total radioactivity exposure (AUC_{0-last}) in tissues of interest and AUC ratio of tissue-to-serum of ^{125}I-protein-01 prepared *via* SIB or Iodogen, after a single IP dose in mice at 8 mg/kg. In general, the exposure of ^{125}I-protein-01 in all the tissues of interest was significantly lower than that in serum. The AUC ratio of tissue-to-serum was less than one, which is consistent with our observations for other antibodies (10). Comparing ^{125}I-protein-01-SIB and ^{125}I-protein-01-Iodogen, one could observe two types of tissue distribution: one with similar AUC values such as for brain, fat, liver, lung, skin, small intestine, spleen and stomach despite the fact that the serum AUC is lower for ^{125}I-protein-01-Iodogen; the other type had similar AUC ratios such as for heart, kidney, large intestine, lymph nodes and muscles, indicating the fast equilibrium

Table III Percentage (%) of TCA Soluble Counts of the Total Radioactivity in Dosing Solution and Mice Serum Samples after Single IP Administration of 8 mg/kg ^{125}I-protein-01 Prepared by Two Iodination Methods and 5 mg/kg SC Injection of ^{125}I-protein-02-SIB (mean ± SD) ($n = 3$)

Protein and labeling method	Dosing solution (%TCA soluble cpm/total cpm)	Route of administration	Serum samples (%TCA soluble cpm/total cpm)							
			0.083 h	1 h	2 h	6 h	24 h	72 h	168 h	336 h
^{125}I-protein-01-SIB	0.1	IP	0.3 ± 0.2	0.4 ± 0.0	ND	0.3 ± 0.1	0.1 ± 0.1	<0.1	<0.1	<0.1
^{125}I-protein-01-Iodogen	2	IP	49.4 ± 29.3	9.7 ± 0.6	ND	16.4 ± 2.3	2.8 ± 0.5	3.1 ± 3.3	29	~100
^{125}I-protein-02-SIB	0.5	SC	27 ± 2.2	ND	2.2 ± 0.4	1.8 ± 0.5	1.1 ± 0.1	2.3 ± 0.1	9.2 ± 0.9	54 ± 3.7

ND not determined

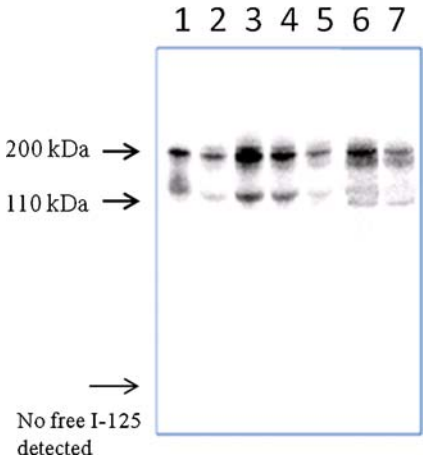

Fig. 4 SDS-PAGE followed by autoradiography analysis of serum samples of ^{125}I-protein-01-SIB. Serum samples were treated by mixing with an equivalent volume of 2× sample buffer, and then being heated at 70°C for 10 min. Lane 1: ^{125}I-protein-01 dosing solution in buffer (as control); *Lane-2 to Lane-7* were serum samples collected at 0.083, 1, 6, 24, 48 and 72 h post injection, respectively. The loaded volume was 6 μL for *Lane-1 to Lane-5*, and 20 μL for *Lanes-6 & 7*.

of ^{125}I-protein-01established between these tissues relative to serum.

DISCUSSION

In this report we evaluated the potential of applying succinimidyl iodobenzoate (SIB) iodination method to label biotherapeutics for studying their PK and biodistribution. Our previous data have demonstrated that the radioisotope probe assay is a cost-effective and fast method to generate informative data for early preclinical PK/ADME studies for biotherapeutics in rodents (10). In general, the radioactive equivalent concentrations per weight of tissue (μg eq./g) are estimated based on the measured total radioactivity in tissues of interest. The accuracy of the assay can be impacted by radioisotope probe stability, such as, a) enzymatic de-halogenation for halogen radioisotope labeling, b) dissociation or proteolytic cleavage of the radioisotope probe from the

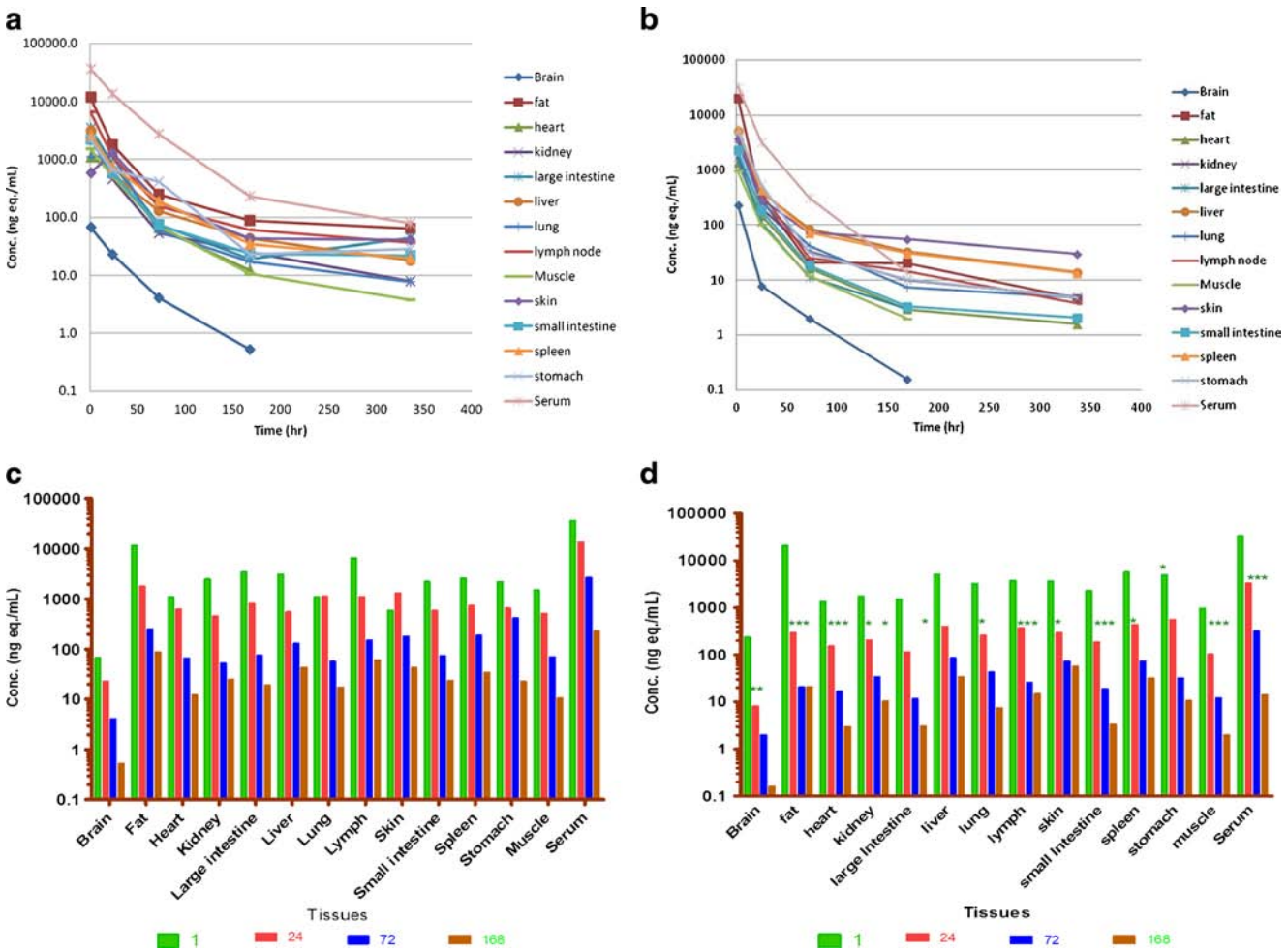

Fig. 5 The radioactive equivalent concentrations (ng eq./g) of ^{125}I-protein-01-SIB (**a** and **c**) and -Iodogen (**b** and **d**) in line and bar graph format, respectively. Tissues of interest at 1, 24, 72, 168, 336 h post IP dosing in mice at 8 mg/kg (mean values with $n = 3$ per time point) were collected and radioactive equivalent concentrations in serum (ng eq./mL) and in tissues of interest (ng eq./g) were calculated based on TCA-precipitable radioactivity for serum and total measured radioactivity for tissues (* statistically different between ^{125}I-protein-01-SIB and ^{125}I-protein-01-Iodogen, $p<0.01$).

Table IV AUC Ratio of Tissue-to-Serum of ^{125}I-Protein-01, Prepared via SIB or Iodogen Iodination Method, in Mice After IP Dosing at 8 mg/kg (Three Mice per Time Point)

	Brain	Fat	Heart	Kidney	Large intestine	Liver	Lung	Lym Jnode	Muscle	Skin	Small intestine	Spleen	Stomach	Serum
$AUC_{0\text{-last}}$[a] (h*μg eq./mL)														
SIB	1.97	227	41	51	77	69	58	133	42	69	54	73	81	1126
Iodogen	3.15	259	23	31	23	83	51	61	16	62	36	90	82	538
AUC ratio of tissue-to-serum														
SIB	0.002	0.20	0.04	0.05	0.07	0.06	0.05	0.12	0.04	0.06	0.05	0.07	0.07	1.00
Iodogen	0.006	0.48	0.04	0.06	0.04	0.16	0.12	0.11	0.03	0.12	0.07	0.17	0.15	1.00

[a] $AUC_{0\text{-last}}$: last t = 168 h post injection; the mean value of total radioactive equivalent concentrations at each time point was used for the AUC calculation

biotherapeutic and c) retention of radioactive proteolytic degradants in tissues. Therefore, initial selection of an appropriate radioisotope and coupling chemistry is critical for PK and ADME studies. Direct iodination methods such as Iodogen and Chloramine-T have been successfully used for the measurement of concentration of biologics in blood and tissue for preclinical PK and ADME studies in many laboratories including ours, (2,5) however, severe *in vivo* instability due to de-iodination was observed in our studies with several biotherapeutic drug candidates. Our experience also showed that, compared to mAbs, de-iodination occurred more often to bifunctional mAbs, mAb fragments and non-mAb therapeutic proteins especially when dosed at low levels with a route extra-venously (Pfizer internal database). Instability of direct iodination in therapeutic proteins has also been reported by other research groups (12,13). The de-iodination event occurs mostly in proteins labeled by the direct labeling method, *e.g.*, Iodogen, and is most likely due to the chemical structure similarities of iodo-phenol to thyroid hormone and wide existence of de-iodination enzymes in blood and tissues (25,27). To overcome such liability, alternative iodination methods need to be exploited in order to improve the accuracy of PK/ADME data generated from the radioisotope probe assays. One approach is the SIB iodination method, where the resulting chemical structure of iodo-benzene differs from iodo-phenol in directly iodinated protein and thyroid hormones. Thus, SIB labeling method could eliminate the enzymatic deiodination of labeled biotherapeutics, improve stability and provide more accurate data, extending the application of radioiodination assays in PK/ADME studies for biotherapeutics. In addition, the bioactivity of biotherapeutics labeled *via* both Iodogen method and SIB method was proven to be retained and comparable (unpublished data).

This investigation has compared PK/biodistribution of protein-01 prepared *via* Iodogen or SIB iodination methods, and that of protein-02 *via* SIB iodination method with unlabeled protein-02 for which concentrations were measured *via* LBA. In these studies, the starting molar ratio of iodine or SIB to protein is ≤1, which would not significantly affect the bioactivity of the labeled proteins. In PK/biodistribution study of protein-01, a significantly higher %free counts in serum, especially at earlier time points, were observed for ^{125}I-protein-01-Iodogen than for ^{125}I-protein-01-SIB (Table III). The %free was determined by TCA precipitation method, a widely used method for PK and biodistribution studies (21) and proteomic analysis where it functions to precipitate stably folded proteins and less well with unfolded, and proteolytically digested proteins (28). Because of the minimal %free counts for ^{125}I-protein-01-SIB, it's believed that the higher %free counts using Iodogen method is due to de-halogenation and not proteolytic degradation of the protein-01. De-halogenation would result in underestimation of the serum and tissue protein concentrations that was reflected by the observed 2 fold decrease in serum AUC (Table II). Since free iodine has a much higher clearance rate thus shorter half-life, about 10 h, than the protein itself (29), the %free counts for ^{125}I-protein-01-Iodogen at later time points decreased more rapidly than the same protein labeled with SIB. Further characterization *via* SDS-PAGE gel/autoradiography demonstrated that the measured equivalent concentration in serum for ^{125}I-protein-01-SIB could fully represent the intact drug concentration (Fig. 4).

^{125}I-protein-02 was also labeled *via* SIB iodination method, and its PK was compared with that of unlabeled protein-02 analyzed by LBA following a single SC administration of 5 mg/kg to normal mice. The serum concentrations of ^{125}I-protein-02-SIB and protein-02-LBA were within 30% variability range of each other and the estimated PK parameters were comparable (Table II). Interestingly, following 5 mg/kg SC administration, %free counts in serum of ^{125}I-protein-02-SIB was 27% compared to 0.3% for ^{125}I-protein-01-SIB through IP administration at 5 min post dose, which is very likely due to the higher protein degradation in subcutaneous tissue after injection than *via* IV or IP administration. At later time points up to 72 h, %free counts was <5% and increased to 9.2% and 54% at 168 and 336 h, respectively. One possible reason for the higher %free counts at the later two time points in this study was due to the low total radioactive counts in the sample, and the inaccuracy due to increased contribution of the background counts.

One of the advantages of using a radioisotope probe assay, such as iodine-125, in ADME studies for biotherapeutics is the relative ease of measuring the drug exposure at tissue target sites, for mechanistic assessment of efficacy and/or off-site binding for potential toxicity, without time-intensive tissue processing or method development. In this study, the mice were pretreated with KI water (20 mM) approximately 3 days prior to dosing to reduce uptake of any potential unbound free ^{125}I generated *in vivo* by organs (thyroid, stomach, kidneys, mammary gland, salivary gland, thymus, epidermis and choroid plexus) containing a sodium-iodide symporter involved in the organification and/or elimination of free ^{125}I (30). Additionally, whole body perfusion was performed to remove the blood content in tissue immediately prior to the tissue harvesting. The radioactive equivalent concentration of ^{125}I-protein-01 in all the tissues of interest was significantly lower than that in serum. Although there was no significant difference in the radioactive equivalent concentration in tissues of interest, except stomach at 1-h post injection for ^{125}I-protein-01 prepared in either iodination methods (Iodogen *vs.* SIB), a higher concentration for ^{125}I-protein-01-SIB than for ^{125}I-protein-01-Iodogen was noticed at later time points (Fig. 5) as observed in serum concentrations. It is interesting to note that there are two types of tissue distribution: one with similar AUC values between ^{125}I-protein-01-SIB and ^{125}I-protein-01-Iodogen such as in brain, fat, liver, lung, skin, small intestine, spleen and stomach; the other type had similar AUC tissue to serum ratios such as heart, kidney, large intestine, lymph nodes and muscles, indicating the fast equilibrium established between these tissues with serum.

As mentioned above, measured total radioactivity is typically used to estimate tissue equivalent concentration for biotherapeutics without further analytical characterization (*e.g.*, SDS-PAGE, LBA or LC-MS/MS), thus data from tissue distribution studies should be interpreted with caution. Based on the studies for ^{125}I-protein-01 and ^{125}I-protein-02, the higher %free counts at early time points, especially using Iodogen method, may likely be due to de-halogenation, in which case the serum and tissue concentrations tend to be underestimated. On the other hand, the potential retention of radioactive degradants of the iodinated biotherapeutics in tissue at later time points tend to overestimate the tissue concentrations. Therefore, it is recommended for ^{125}I labeled protein biodistribution studies, the time points are designed no longer than 168 h post dose unless additional assays will be conducted to quantify the intact proteins.

CONCLUSION

The SIB radioiodination method by conjugating the intermediate of N-succinimidyl [^{125}I]iodobenzoate onto the amine group of the lysine residues in biotherapeutics can eliminate the radiolabel stability issue occurring in direct iodination methods thus the PK parameters acquired are more accurate than acquired *via* direct iodination methods. In conclusion, SIB iodination chemistry provides an alternative and improved method for the assessment of PK/ADME properties of therapeutic proteins.

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