Kim *et al.*
Journal of Analytical Science and Technology (2024) 15:6
https://doi.org/10.1186/s40543-024-00420-w

Journal of Analytical Science and Technology

RESEARCH ARTICLE

Open Access

Method development for ^{14}C-labeling of IgG antibodies in preparation for clinical trials

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Abstract

Objectives Carbon-14 (^{14}C) labeling is a standard technology for tracing molecules and providing their pharmacokinetic profiles. However, its primary focus has been on small molecules, with limited application to biomacromolecules. Particularly in the development of new biological entities (NBE), the utilization of microdosing with a ^{14}C-labeled biomacromolecule proves beneficial in the early stages of drug development, contributing to significant time and cost savings. This study investigates the ^{14}C-labeling of antibody and explores the stability of ^{14}C-labeled antibody under various storage conditions.

Methods and results In this study, the utilization of ^{14}C-formaldehyde for labeling target antibodies at various molar ratios revealed a direct correlation between labeling efficiency and the quantity of ^{14}C-formaldehyde applied: 1.5 mol/mol for ^{14}C-labeled antibody with the use of 10 equivalents of ^{14}C-formaldehyde, 3.8 mol/mol for ^{14}C-labeled antibody with the use of 10 equivalents of ^{14}C-formaldehyde, and 10.5 mol/mol for ^{14}C-labeled antibody with the use of 60 equivalents of ^{14}C-formaldehyde. All the reaction conditions exhibited no antibody degradation, as evidenced by the absence of a significant change in HPLC purity compared to the unlabeled antibody. Stability tests revealed that all groups maintained their purities over a 4-week period at both -75 ± 10 °C and 5 ± 3 °C. Given safety concerns related to internal radiation exposure in potential human subjects during microdosing, this study established optimal conditions for employing ^{14}C-labeled antibodies. Therefore, it is optimized that 10 equivalents of ^{14}C-formaldehyde can be used for ^{14}C-antibody labeling through reductive amination, storing the antibodies at 5 ± 3 °C, and assigning a storage period of 4 weeks.

Conclusion The findings from this study offer valuable insights into the effective application of ^{14}C-labeling in microdosing studies, especially for larger molecules such as antibodies.

Keywords ^{14}C-labeling, Reductive amination, Antibody, Microdosing, ^{14}C-formaldehyde

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Introduction

Radioisotope-labeled compounds are utilized to monitor their biological and physicochemical behaviors by detecting their radioactivity. These compounds find application in pharmacokinetics (PK) analysis of drug candidates during the new drug development process. Their ability to reproduce the same metabolic processes in vivo while preserving the chemical structure and characteristics of common compounds makes them valuable tools in drug development (Bae and Shon 2011). In general, new drug development takes 10–15 years or more and costs more than $1 billion. Approximately 60% of the total cost is invested in clinical trials, but the success rate is less than 10% (Paul et al. 2010). The main reason for the low success rate of clinical trials is that the development of a new drug is primarily based on experimental approaches, either in vitro or in vivo. More recently, attempts have been made to conduct in silico trials for establishing safety and efficacy evidence through modelling and simulation. However, the obtained results occasionally diverge from clinical PK results, posing challenges in sufficiently demonstrating safety and efficacy (Viceconti et al. 2021). To address this issue, the radioisotope-accelerator mass spectrometry (RI-AMS) method investigates the absorption, metabolism, and excretion of drug candidates in the human body using compounds labeled with radioisotopes such as carbon-14 (Dalvie 2000). In particular, biological drugs face challenges due to the lack of predictive preclinical models for studying PK profiles. Clinical trials applying RI-AMS to humans are referred to as microdosing. Microdosing, conducted prior to standard clinical trials, involves administering a smaller dose (typically 1/100 of the therapeutic dose) to the human body. Given the biomacromolecule new drugs compared to small molecule drugs, the dosage in clinical trials is less than 30 nmol. This approach allows for the acquisition of precise PK data on drug distribution and metabolism, assessing their reach to specific molecular targets while minimizing the risk of radiation exposure and toxicity associated with new drugs. This methodology guides the selection of drug candidates in the early stages of development. Moreover, from an economic standpoint, drugs used in microdosing do not necessitate Good Manufacturing Practice (GMP) manufacturing required for general clinical trials. This positive aspect contributes to reducing the preparation cost for clinical trials as much as possible (Lappin et al. 2006; Vlaming et al. 2015).

When PK data is obtained through liquid scintillation counting (LSC) pharmacokinetic analysis during microdosing, a radioactive amount of 0.74–3.7 MBq is usually administered to the human body. In contrast, while utilizing accelerator mass spectrometry (AMS) analysis, the administered radioactive dose is reduced to a range of 0.0037–0.037 MBq (Roffel et al. 2016; Spracklin et al. 2020). AMS, compared to LSC analysis technology, offers high-sensitivity quantification and rapid analysis, providing a notable advantage in significantly minimizing the amount of radioactive material administered during microdosing. This ensures the safety of test subjects by reducing the risk of internal radiation exposure (Bae and Shon 2011; Burt et al. 2016; Vogel et al. 2011; Vuong et al. 2015). AMS analysis demonstrates relatively higher sensitivity and accuracy in microdosing studies (Garner et al. 2000; Keck et al. 2010). Additionally, antibody drugs play a significant role in the pharmaceutical market as biological products, with the market share of biopharmaceuticals expected to expand in the future due to the development of innovative antibody drugs such as antibody–drug conjugates and bispecific antibodies (Carter and Lazar 2018; Schuurman and Parren 2016). Consequently, the importance of ^{14}C-labeled compounds and labeling technology is on the rise and is anticipated to further increase to facilitate the swift market entry of new antibody drugs.

The introduction of ^{14}C-labeling technology for low-molecular-weight substances primarily involves organic reactions (Babin et al. 2022). However, biomolecules such as proteins and antibodies, characterized by their large molecular weight and multiple charges, pose greater challenges for ^{14}C-labeling due to their limited solubility in organic solvents compared to small molecules (Edelmann 2022). Antibodies or proteins labeled with radioisotopes may undergo abnormal transformation due to the influence of radiation energy depending on the manufacturing process or storage method, and modified antibodies are less active than regular antibodies (Lappin and Garner 2003; Ma et al. 2020).

In 2015, the initial revelation of microdosing test data involving ^{14}C-labeled recombinant human proteins marked a significant milestone (Vlaming et al. 2015). Subsequently, in 2018, a nanotracing test for antibodies was documented (Kratochwil et al. 2018). Nevertheless, in contrast to the research and development progress made with small molecule compounds, the exploration of ^{14}C-labeling and microdosing tests for antibodies and proteins remains a challenging frontier (Burt et al. 2020).

As a late-stage ^{14}C-labeling, *N*-succinimidyl[1-^{14}C]propionate ([^{14}C]NSP), has been employed for ^{14}C-labeling antibody (Kratochwil et al. 2018). Notably, [^{14}C]CH_2O (formaldehyde), has primarily been utilized for small molecule labeling through hydromethylation of unactivated olefins.

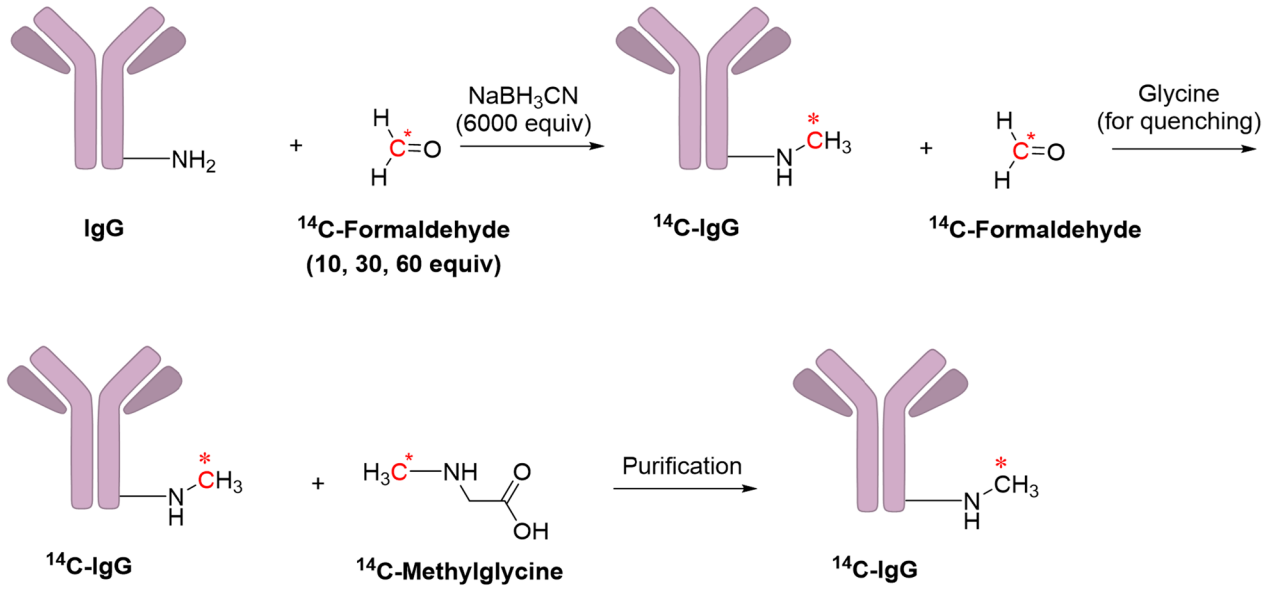


Fig. 1 Schematic view of ^{14}C-labeling of IgG antibody and purification using ^{14}C-formaldehyde and $NaBH_3CN$

To the best of our knowledge, this is the first report on the technique of ^{14}C-labeling antibodies using ^{14}C-formaldehyde. Consequently, it becomes imperative to devise a methodology surpassing the efficacy of alternative radioisotope labeling methods. Our study addresses the constraints associated with labeling reagents and reporting by introducing a novel method for preparing ^{14}C-labeled antibodies (Fig. 1).

In this report, we present an innovative approach involving antibody labeling with ^{14}C-formaldehyde through reductive amination. The research was designed as follows: Firstly, the general reductive amination approach to protein labeling has been reported in Means and Feeney (1968). We applied the same method using ^{14}C-formaldehyde, anticipating ^{14}C-labeling on Lys residues. Secondly, we optimized the reaction conditions through molar ratio variations, achieving 44–50% recovery yields while maintaining antibody affinity. The reaction with 10 equivalents of reagents demonstrated a labeling efficiency of 1.5 mol/mol. Thirdly, we conducted stability tests on the ^{14}C-labeled antibody. Results revealed that antibodies produced using the ^{14}C-labeled method developed in this study remained stable for up to 4 weeks at -75 ± 10 °C and 5 ± 3 °C. Finally, optimal conditions for applying ^{14}C-labeled antibodies to clinical trials were identified as reaction with 10 equivalents of ^{14}C formaldehyde and storing them at 5 ± 3 °C for a duration of 4 weeks.

Results

Determination of ^{14}C-labeling efficiency

In this study, anti-mouse rabbit IgG was chosen as an example of IgG antibody. Reaction conditions were explored based on the molar equivalent of the ^{14}C-formamide reagent. Using an excess amount (6000 equiv) of $NaBH_3CN$ and 10, 30, and 60 equiv of ^{14}C-formaldehyde were reacted with anti-mouse rabbit IgG (Fig. 1). Each reaction of ^{14}C labeling on anti-mouse rabbit IgG were analyzed and optimized the reaction condition for ^{14}C-antibody labeling. The concentration of ^{14}C-formaldehyde was determined by using a chromotropic acid indicator (Dar et al. 2016; Jendral et al. 2011). A standard curve was constructed, and a function derived from the formaldehyde concentration of the standard solution and each absorbance value (A580 nm), resulting in the equation $y = 0.0674x - 0.0258$, and a correlation coefficient (R^2) of 0.989 (Fig. 2). The concentration of ^{14}C-formaldehyde was determined to be 5.24 mg/mL (0.16 mmol/mL). The protein concentration of ^{14}C-anti-mouse rabbit IgG was determined using the Bradford assay.

The radioactivity concentration, calculated from the dpm values measured through LSC, was found to be 0.0056 MBq/mL for the ^{14}C-anti-mouse rabbit IgG when 10 equiv of ^{14}C-formaldehyde was used (Fig. 3a). The radioactivity concentrations were 0.014 and 0.036 MBq/mL, when 30 and 60 equiv of ^{14}C-Formaldehyde were

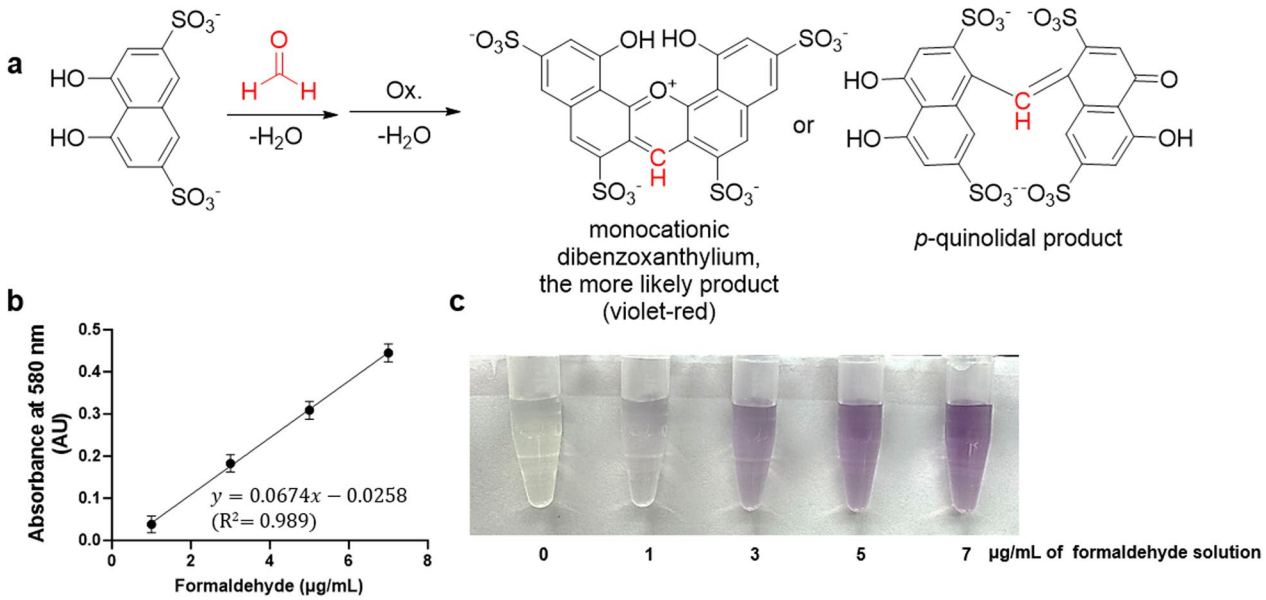


Fig. 2 Determination of formaldehyde concentration using chromotropic acid. **a** Hypothetical reaction products of the chromotropic acid reacted with formaldehyde in strongly acidic media. The monocationic dibenzoxanthylium is the more probable product, although the chemistry is intricate and not yet fully understood. **b** calibration curve using standard solution. **c** Example images depicting reactions with standard solutions

used, respectively. By calculating the specific radioactivity using the protein concentration and the radioactivity concentration, the specific radioactivity of ^{14}C-anti-mouse rabbit IgG was determined to be 0.022, 0.059, and 0.16 MBq/mg, when 10, 30 and 60 equiv of ^{14}C-formaldehyde were used. Finally, the ^{14}C labeling efficiencies were calculated based on the ^{14}C labeling radioactivity, using the maximum specific radioactivity value (62.4 mCi/mmol or 2308.8 MBq/mmol) of ^{14}C isotope (Babin et al. 2022). As a result, labeling efficiencies of ^{14}C-anti-mouse rabbit IgG of each reaction condition were calculated as 1.5, 3.8 and 11 mol/mol for 10, 30 and 60 equiv of ^{14}C-formaldehyde were used, respectively (Fig. 3a, b). The antibody activity was demonstrated to be retained, as assessed by the dot blotting method (Fig. 3c, d).

$$Radioactive\ Conc.\left(\frac{\mathrm{MBq}}{\mathrm{mL}}\right)=\frac{radioactivity\ (\mathrm{dpm})}{60\left(\frac{\mathrm{dpm}}{\mathrm{Bq}}\right)\times 10^{6}\times sample\ vol.\ (\mathrm{mL})} \tag{1}$$

$$Labeling\ Efficiency\cdot\left(\frac{mole}{mole}\right)=\frac{S.A.\left(\frac{MBq}{mg}\right)}{2308.8\left(maximum\ specific\ activity\ of\ ^{14}C,\frac{MBq}{mmol}\right)\times 150,000\left(Molecular\ Weight,\frac{mg}{mmol}\right)} \tag{2}$$

Determination of chemical and radiochemical purity and the antibody stability

Chemical and radiochemical purity assessments were conducted for all samples containing ^{14}C-IgG under various reaction conditions, ensuring the integrity of the antibody and absence of degraded impurities (Fig. 4a, b). The comparison of retention times between IgG and ^{14}C-IgG in both the UV chromatogram and radioactive chromatogram verified the alignment of peaks at the same retention time, confirming the presence of ^{14}C-IgG. To assess the stability of 14C-antibody, a 4-week evaluation was conducted at two temperature conditions, -75 ± 10 °C and 5 ± 3 °C. For each ^{14}C-IgG with different labeling efficiencies, the peak area of IgG detected at 220 nm was analyzed over this period. The standard deviation value divided by the average value was expressed as a percentage and compared, as depicted in Fig. 4e. Results suggest that ^{14}C-IgG remains more stable for up to 4 weeks at 5 ± 3 °C compared to -75 ± 10 °C. However, the stability of the antibody was observed to be unaffected by varying labeling efficiencies.

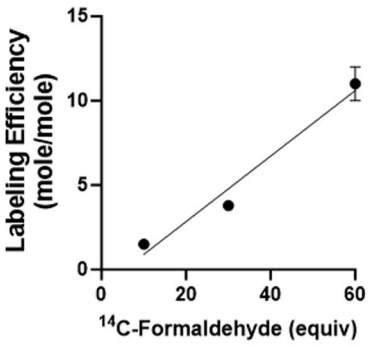
Discussion

In this study, the reductive alkylation method for labeling proteins (Vlaming et al. 2015) was applied to ^{14}C-labeling of antibodies using ^{14}C-formaldehyde. During the development of a method for labeling IgG

a

^{14}C-formaldehyde (equiv)	Protein Conc. (mg/mL)	Radioactive Conc. (MBq/mL)	Specific Activity (S.A.) (MBq/mg)	Labeling Efficiency (mole/mole)
10	0.25 ± 0.01	0.0056 ± 0.0001	0.022 ± 0.001	1.5 ± 0.1
30	0.24 ± 0.01	0.014 ± 0.001	0.059 ± 0.001	3.8 ± 0.1
60	0.22 ± 0.01	0.036 ± 0.001	0.16 ± 0.01	11 ± 1

b



c

^{14}C-formaldehyde (equiv)	Antibody Activity of IgG (ng)			
	0	0.1	1	10
0				
10				
30				
60				

d

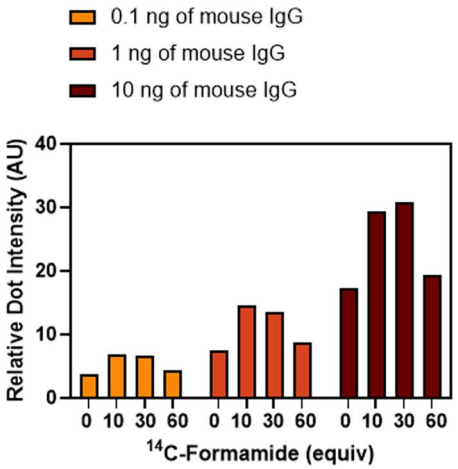


Fig. 3 Protein concentration, radioactive concentration, specific activity, ^{14}C-labeing efficiencies, and antibody activity test. **a** Protein concentration determined by Bradford assay, radioactive concentration measured by LSC, specific activities and ^{14}C-labeing efficiencies calculated by following two formulas (1 and 2). **b** Plot between labeling efficiency and ^{14}C-formamide molar equiv. **c** Antibody activities detected by the dot blot method. Mouse IgG was used as an antigen and concentration dependent antigens were detected by ^{14}C-labeled anti-mouse rabbit IgG with different labeling efficiencies. Alkaline Phosphatase conjugated anti-rabbit goat IgG was used as the second antibody. Each antibody concentration was estimated by the intensity of dot blot. **d** The dot intensities were analyzed by ImageJ software (National Institute of Health (NIH), Bethesda, USA), visually represented as indicated, and then compared with the antibody activity in each reaction condition

antibodies with the ^{14}C-isotope, the antibody recovery yield for the ^{14}C-labeling process was determined to be 44–50%. Employing 10, 30, and 60 equiv of ^{14}C-formaldehyde based on anti-mouse rabbit IgG, 60 equiv of ^{14}C-formaldehyde exhibited the highest labeling efficiency as 11 mol/mol, surpassing that of 10 equiv of ^{14}C-formaldehyde used by 7 times with labeling efficiency of 1.5 mol/mol. The analysis of ^{14}C-labeling revealed that the ^{14}C-labeling yield is directly proportional to the amount of ^{14}C-formaldehyde, suggesting the feasibility of synthesizing antibodies with the desired specific radioactivity by adjusting the equivalent amount of ^{14}C-formaldehyde. Nevertheless, the challenge of achieving regiospecific labeling without random reductive amination on Lys side chains persists, although potential solutions may lie in previous reports detailing selective modifications such as the N-terminal region-selective labeling method (Scheck and Francis 2007). After ^{14}C-labeling, all ^{14}C-IgG with the different labeling efficiencies under the different reaction conditions demonstrated close to 100% chemically and radiochemically pure, further confirming the

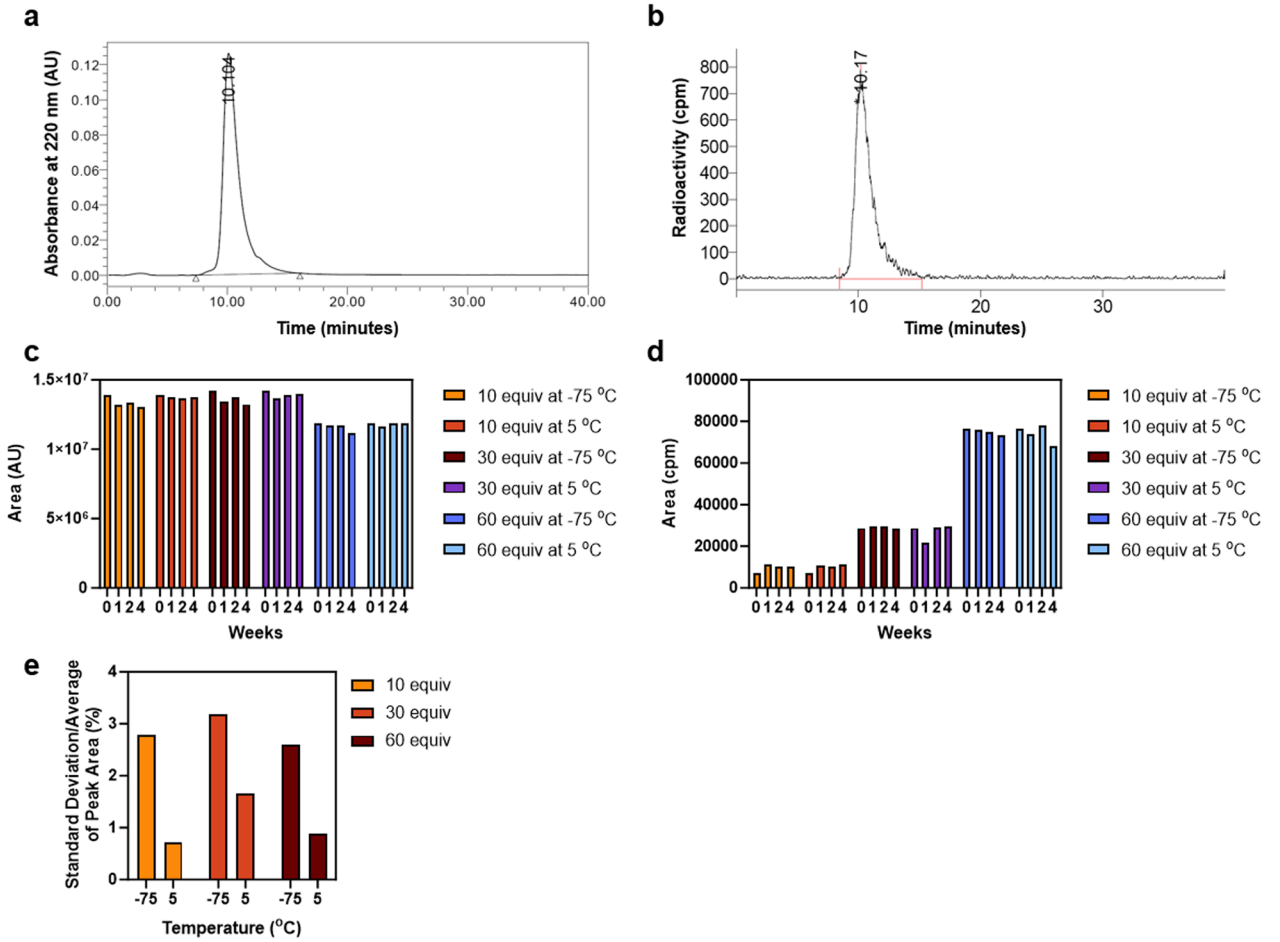


Fig. 4 Purity and stability of ^{14}C-IgG. **a** Representative UV chromatogram of the 14C-IgG. **b** Representative radioactive chromatogram of the 14C-IgG. **c** Plot of peak area detected by UV absorbance at 220 nm over 4 weeks at two different temperatures, 75 ± 10 °C and 5 ± 3 °C. **d** Plot of peak area detected by radioactivity (cpm) over 4 weeks at two different temperatures, 75 ± 10 °C and 5 ± 3 °C. **e** Relative stability (%) over 4 weeks expressed as the standard deviation divided by the average of each condition

same activity with the unlabeled IgG. Results indicate that there is no denaturation of IgG antibody detected even after reductive alkylation reaction with the amine group ($-NH_2$) such as Lys residues. Based on these findings, it is inferred that the same labeling method can be extended to other proteins and antibodies possessing Lys residues. In the stability test, ^{14}C-IgG antibody was confirmed to remain stable for up to 4 weeks at −75 ± 10 °C and 5 ± 3 °C. The more detailed analysis showed that the ^{14}C-IgG antibody using this reductive alkylation is more stable at 5 ± 3 °C than at −75 ± 10 °C. In a previous report, microdosing was conducted using ^{14}C-human anti-IL-17 IgG1 with a specific radioactivity value of 0.036 MBq/mg and a labeling efficiency of 2.3 mol/mol (Kratochwil et al. 2018). Given the extended half-life of IgG in the human body (25.8 days), it is crucial to address safety concerns pertaining to internal radiation exposure in potential human subjects (Kontermann 2016; Mankarious et al. 1988). Therefore, microdosing with ^{14}C-IgG produced under the condition of utilizing 10 equiv of ^{14}C-formaldehyde (with a labeling efficiency of 1.5 mol/mol of and a specific activity of 0.023 MBq/mg) was deemed the most economical and rational approach to ensure safety against internal radioactive exposure to the test subjects in clinical trials.

Conclusions

This study reveals that the optimal conditions for applying ^{14}C-labeled antibodies to clinical trials involve reductive amination with 10 equiv of ^{14}C-formaldehyde and storing at 5 ± 3 °C for a duration of 4 weeks. Additionally, the utilization of ^{14}C-labeling and microdosing

tests on various new protein drugs and antibody drugs with substantial molecular weight is anticipated to yield more accurate pharmacokinetic data in the human body, contributing significantly to cost and time savings in the early stages of drug development.

Methods

^{14}C-labeling of IgG antibody and purification

The reductive alkylation method for protein labeling using ^{14}C was applied in this experiment. (Means and Feeney 1968). Anti-mouse rabbit IgG (Sigma-Aldrich, AP160) antibody was chosen as an antibody sample. One milligram of the antibody was reacted with 10, 30 and 60 equiv of ^{14}C-formaldehyde aqueous solution (CuraChem Inc., Formal-200, 340 MBq/mL of radioactivity conc., specific activity of 2.11 GBq/mmol (65.88 MBq/mg) CuraChem Inc., authorized under license (License No. 13-1147-4) issued by the Nuclear Safety and Security Commission of the Republic of Korea, produced ^{14}C-formaldehyde.). The reaction took place in a 2 mL buffer solution (reaction buffer, 0.01 M sodium phosphate, 0.25 M sodium chloride, pH 7.6) with stirring at 1000 rpm at room temperature for 10 min. Subsequently, a 1 M sodium cyanoborohydride ($NaBH_3CN$) solution (40 µL, 6000 equiv) was added in three portions to initiate the reaction, which proceeded for 30 min. Upon completion, a 10% (w/v) glycine aqueous solution (30 µL, 6000 equiv) was introduced and stirred for 10 min to quench the reaction.

Following the reaction, a desalting column was used to eliminate the excess amount of unreacted ^{14}C-formaldehyde and reagents. Briefly, 2 mL of the reaction mixture was loaded on a PD-10 column (GE Healthcare, 17085101), with the initial eluate discarded. Subsequently, 0.5 mL of a sodium phosphate storage buffer solution (same as the reaction buffer above) was added to the PD-10 column, and after discarding the eluate, 3.5 mL of storage buffer solution was introduced to recover the eluate, effectively separating the antibody from the reaction reagent. The solution, purified through the PD-10 column, underwent further processing in a 30 kDa cut-off centrifugal concentrator (Amicon, UFC903024) for buffer solution exchange. Specifically, 3.5 mL of the antibody solution obtained through PD-10 column purification was placed in the centrifugal concentrator and concentrated by centrifugation at 4000 rpm and 4 °C for 20 min. After concentration, 10 mL of buffer solution was added, and the concentration process was repeated three times in the same manner. Antibody in a buffer solution was subsequently concentrated to achieve a total volume of 2 mL.

Determination of ^{14}C-formamide concentration

The concentration of ^{14}C-formaldehyde was determined by measuring absorbance using a chromotropic acid indicator (Dar et al. 2016). Briefly, a 1% chromotropic acid indicator solution (w/v, SigmaAldrich 126225) was prepared and filtered through a 0.45 µm syringe filter before use. Absorbance at 580 nm was obtained using a UV–Vis spectrophotometer (KLAB, OPTIZEN Alpha). To create a formaldehyde standard solution, 37% formaldehyde (SigmaAldrich 252549) was diluted in water to achieve concentrations of 1, 3, 5 and 7 µg/mL. The ^{14}C-formaldehyde was then diluted 1000 times, and a subsequent 5000-fold dilution was analyzed based on the standard curve.

For measuring formaldehyde concentration, 400 µL of standard solution or analysis solution was mixed by stirring with 10 µL of 1% chromotropic acid. Subsequently, 600 µL of 35% sulfuric acid solution (w/v) was added, and the mixture was stirred. The mixture was incubated for 15 min in a heating block preheated to 95 °C, followed by standing at room temperature for 1 h. Finally, the absorbance (A580 nm) was measured.

Determination of specific activity (S.A.)

Using the protein concentration and radioactivity concentration values of ^{14}C Anti-mouse rabbit IgG, the specific radioactivity value was calculated according to the formula (3). The radioactivity was measured by mixing 0.1 mL of ^{14}C-anti-mouse rabbit IgG solution with 10 mL of LSC cocktail (Perkin Elmer 6013319) in a liquid scintillation counter (LSC).

$$^{14}C\ IgG\ specific\ activity\ (S.A.,\ MBq/mg) = \frac{\text{Radioactivity of } ^{14}C\ IgG\ (\text{dpm})}{\text{Conc. of IgG}\left(\frac{\text{mg}}{\text{mL}}\right) \times sample\ vol.\ (\text{mL}) \times 60\left(\frac{\text{dpm}}{\text{Bq}}\right) \times 10^6} \quad (3)$$

Determination of antibody concentration

Protein concentration was determined by Bradford method based on the Coomassie Brilliant Blue dye. Briefly, 300 µL of each standard solution and ^{14}C-anti-mouse rabbit IgG were placed in a reaction tube, followed by the addition of 300 µL of Coomassie reagent (ThermoFisher, 23200) and thorough mixing. A standard curve relating protein concentration to absorbance (A595 nm) was established using 3.125, 6.25, 12.5 and 25 µg/mL of BSA solution. The ^{14}C-anti-mouse rabbit IgG solution was diluted 25 times with a buffer solution, and its concentration was measured. The reaction solution was

allowed to react at room temperature for 10 min, and then the absorbance (A595 nm) was measured. Protein concentration in the ^{14}C-anti-mouse rabbit IgG solution was subsequently calculated by using the standard curve.

HPLC chromatogram

HPLC analysis was performed for the identification and quantitation of impurities in ^{14}C-labeled antibody. The purity of ^{14}C-Anti-mouse rabbit IgG was assessed using a Waters Alliance e2695 high-performance liquid chromatography (HPLC) system. The HPLC trace of ^{14}C-anti-mouse rabbit IgG confirmed the integrity of antibody by comparing the retention time of anti-mouse rabbit IgG. In brief, 50 µL of a 0.5 mg/mL antibody solution in a buffer of 0.01 M sodium phosphate, 0.25 M sodium chloride (pH 7.6) was loaded onto a TSKgel® G3000 SW_{XL}, HPLC column (7.8 mm × 300.0 mm, 5 µm particle size, Tosoh 0008541) at 25 °C equipped with a guard column, TSKgel® G3000 SW_{XL} Type Guard Column (6 mm × 40 mm, 7 µm particle size, Tosoh 0008543). Protein peaks were detected at 220 nm coupled with a radio detector, Berthold Flowstar^2 LB513. Samples were mixed with liquid scintillation cocktail, FLO-SCINT™ II (PerkinElmer 6013529), using a liquid scintillation pump at a flow rate of 3.0 mL/min. The mobile phase consisted of a buffer composed of 0.1 M Sodium phosphate and 0.2 M Sodium chloride, pH 6.8, at a flow rate of 1 mL/min.

Antibody activity test

The antibody activity was evaluated using the dot blotting method (Rupprecht et al. 2010). Standard solutions of mouse IgG (Sigma-Aldrich NI03) at a concentration of 100 µg/mL were prepared by diluting in Tris-Buffered Saline, 0.1% Tween® 20 detergent (TBST) to concentrations of 0.1, 1 and 10 µg/mL. For the assay, 1 µL of each concentration of the mouse IgG standard solution was directly spotted on a western blotting PVDF membrane (Sigma-Aldrich 3010040001) and allowed to dry at room temperature for 10 min. Subsequently, 5 mL of 10% BSA in TBST buffer solution (w/v) was added to the dried membrane, and it was allowed to react at room temperature for 1 h. After removing the 10% BSA in TBST buffer solution, 0.48 µg/mL anti-mouse rabbit IgG, diluted in 10% BSA buffer solution, was added to the membrane and allowed to react at room temperature for 16 h. Following this, the anti-mouse rabbit IgG solution was removed, 10 mL of TBST buffer solution was added to the membrane, and the membrane was washed for 10 min—this washing process was repeated three times. Subsequently, 5 mL of anti-rabbit goat IgG (Alkaline Phosphatase conjugate, ThermoFisher 31340), diluted in TBST buffer solution to a concentration of 0.48 µg/mL, was added to the membrane and allowed to react at room temperature for 1 h. Similar to the previous steps, the anti-rabbit goat IgG solution was removed, 10 mL of buffer solution was added to the membrane, and the membrane was washed for 10 min, repeated three times. Following the washing steps, the buffer solution was removed, and 1 mL of 5-bromo-4-chloro-3-indolyl-phosphate/Nitro blue tetrazolium chloride solution (BCIP/NBT solution, Sigma-Aldrich B1911) was added. Color development occurred at room temperature for 20 min. The BCIP/NBT solution was then removed, 10 mL of water was added to the membrane, and the membrane was washed for 10 min. The color intensity of each ^{14}C-Anti-mouse rabbit IgG 14-C-labeled antibody with the use of 10, 30, and 60 equiv of ^{14}C-formamide was visually compared with the results of the anti-mouse rabbit IgG control test. ImageJ software (National Institute of Health (NIH), Bethesda, USA) was employed to analyze the intensity of each dot and compare the antibody activity.

Abbreviations

IgG	Immunoglobulin G
PK	Pharmacokinetics
GMP	Good manufacturing practice
LSC	Liquid scintillation counting
AMS	Accelerator mass spectrometry
rpm	Revolutions per minute
S.A.	Specific activity
dpm	Disintegrations per minute
UV	Ultra-violet
BSA	Bovine serum albumin
HPLC	High performance liquid chromatography
Conc.	Concentration
R^2	Correlation coefficient
equiv	Equivalent

Acknowledgements

Not applicable.

Author contributions

SK, JHK, SH and JKJ analyzed the data and wrote the manuscript. SK performed the experiments. JHK, SH and JKJ conceptualized and designed the study. All authors read and approved the final manuscript.

Funding

This study was supported by the National Research Foundation of Korea (NRF-2021R1I1A1A01059834, MRC 2017R1A5A2015541). This research was supported by "Regional Innovation Strategy (RIS)" through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (MOE) (2021RIS-001). This work was supported by the research grant of the Chungbuk National University in 2021.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Competing interests

The authors declare that they have no competing interests.

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Received: 10 January 2024 Accepted: 14 February 2024
Published online: 27 February 2024

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