Direct radio-iodination of folic acid for targeting folate receptor-positive tumors

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ABSTRACT

The folate receptor (FR) is a promising cell membrane-associated target for nuclear imaging of various cancers (via imaging FR-α) and potentially also inflammatory diseases (via imaging FR-β), through the use of folic acid-based radioconjugates. However, there have been several drawbacks of previously reported radioconjugates, such as a short half-life of the radiolabel (^{68}Ga $t_{1/2}$ 68 min), a complex and time-consuming multistep radiosynthesis, and a high renal uptake of radiolabeled folate derivatives. The goal of this study was to develop an imaging probe by directly labeling folate with radioactive iodine without using an extra prosthetic group. The radiolabeling of folate was optimized using various labeling conditions and the labeled tracers were isolated by high-performance liquid chromatography. The *in vitro* stability of labeled folate was checked in phosphate-buffered saline and serum. The tumor-targeting efficacy of the probe was also evaluated by biodistribution studies using a murine 4T1 tumor model.

Key Word: Folic acid, radioiodination, tumor targeting

Introduction

Folic acid, also known as vitamin B9, is an essential compound that is involved in several important biochemical processes in humans, such as cell proliferation, gene regulation, and red and white blood cell production (1). It is widely considered an ideal targeting agent for therapeutic and diagnostic imaging of tumors owing to its high affinity ($K_D < 10^{-9}$ M) to both folate receptor-α and folate receptor-β, which are highly over-expressed in solid tumors and negligibly expressed in the majority of normal tissues (2, 3, 4).

For instance, their expression was positive in almost 89% of human ovarian carcinomas but was minor in both mucinous ovarian carcinomas and normal ovary (4). Moreover, the level of FR-β expression on macrophages involved in inflammatory processes was determined as an activation marker for macrophages (5). The molecular structure of folate is constituted of three parts, namely a glutamic acid (Glu) moiety, a p-aminobenzoic acid moiety (PABA), and a pterin moiety (6) (Fig. 1), and its structure can be easily modified to develop folate-based radiotracers. There are numerous folate conjugates for nuclear imaging

via positron emission tomography and single-photon emission computed tomography, such as [66/67/68]Ga-deferoxamine-folate (7), 2′-^{18}F-fluorofolic acid (8), and ^{111}In-diethylenetriaminepentaacetic acid-folate (9). According to Dr. Josefine Reber, the main drawback of radiofolates is their high renal uptake, which represents a barrier for their therapeutic application (10). In the research by Dr. Reber's group, the folate conjugates tyrosine-folate and tyrosine-click-folate were synthesized and radioiodinated by the iodogen method. Free iodide ions de-iodinated from the compound increased the uptake of the radiolabel by the thyroid and reduced its uptake by the kidneys (10). We wondered whether it would be possible to directly radiolabel folic acid with radioiodine without extra conjugation. The aim of this study was to optimize a radioiodolabeling method for direct radioiodination of folic acid, and thereafter evaluate the *in vitro* stability, *in vivo* behavior, and selective tumor targeting of the radiolabeled folic acid probe via biodistribution experiments.
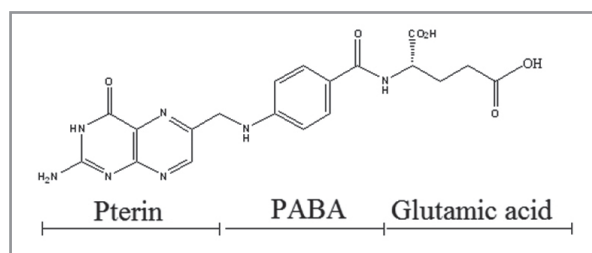
Figure 1. Chemical structure of folic acid.

Materials and Methods

Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), including folic acid (≥97% purity), Iodobeads®, iodic acid (HIO₃, ACS reagent, ≥99.5%

purity), and trifluoroacetic acid (TFA; reagentPlus®, 99% purity). Acetonitrile (high-performance liquid chromatography [HPLC] grade) was purchased from Duksan Pure Chemicals (Ansan, Korea). Deionized distilled water (Milli-Q® Gradient, Millipore, Billerica, MA) for the mobile phase was acidified with TFA (0.1%). Phosphate-buffered saline (PBS) was obtained from Hyclone (Logan, UT). All other chemicals were of analytical grade.

Chromatographic conditions

Identification and purification of radiolabeled folic acid compounds were achieved using a HPLC system equipped with ultraviolet and radio detectors (Waters, Milford, MA). A mobile phase of water (0.1% TFA)/acetonitrile (0.1% TFA) was used with a gradient elution from 0–30% acetonitrile (0.1% TFA) over 20 min with a flow rate of 1 mL/min. The mobile phase solutions were vacuum-filtered through 0.45-μm nylon membranes and carefully degassed before usage. The absorbance of the eluent was measured and folic acid was detected at a wavelength of 290 nm. An XBridge RP18 column (150 × 4.6 mm, 5 μm; Waters) was used for the separation of radiolabeled compounds.

A radio thin-layer chromatography (radio-TLC) scanner was used to monitor the radiolabeling yield of ^{131}I-folic acid under different reaction conditions. The stationary phase was silica and the mobile phase was EtOAc:MeOH:H₂O (5:3:2).

Radiochemistry

Procedure 1: Using the iodobead method

A 2.28-mg/mL stock solution of folic acid was prepared in MeOH:water (1:1). One iodobead was was washed with 500 μL PBS to remove any loose particles and remnant reagents. The bead was dried on filter paper. In a reaction tube, 100 μL PBS was pre-incubated with 1 iodobead and 3.7 MBq of ^{131}I for 5 min. Then, 10 μL folic acid (22.8 μg) was added into the tube and the mixture shaken at 850 rpm for 1 h at 30 or 60 °C. After that, the bead was removed from the solution to terminate the reaction.

Procedure 2: Using iodic acid HIO$_3$ iodic acid HIO$_3$ method

Folic acid (22.8 μg, 10 μL) was added to 100 μL of PBS, mixed with Na^{131}I (3.7 MBq) and HIO$_3$ (10 μL) 0.1 M, and the mixture shaken at 850 rpm for 1h at 30 and 60 °C. The reaction progress was monitored by radio-TLC and HPLC.

Evaluation of serum stability

For in vitro stability studies, approximately 3.7 MBq of the radiolabeled compound ^{131}I-folic acid, purified by HPLC, was added to either 500 μL of PBS or 500 μL of human serum. The resulting mixtures were incubated at 37 °C with shaking at 750 rpm. The radiolabeled compounds were detected by radio-TLC (silica, EtOAc:MeOH:H$_2$O [5:3:2]) at various time points (0–24 h).

Biodistribution studies

All animal experiments were conducted in compliance with the requirements of the Animal Care and Use Committee of Kyungpook National University. 4T1 breast tumor cells were cultured RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a 5% CO$_2$ atmosphere. Female BALB/c mice at 9 weeks of age were obtained from Hyochang Science (Daegu, Korea). The mice were injected subcutaneously in the right femoral region with 5×10^6 4T1 cells suspended in 100 μL RPMI 1640 medium. When the tumors reached 9–10 mm diameter (7–10 days after implantation), the mice were used for biodistribution studies. The mice were injected with ^{131}I-folic acid (~ 0.74 MBq) via the tail vein.. After the appropriate uptake time (1 or 4 h after injection), anesthesia was induced with 2% isoflurane and then the mice were exsanguinated. Tissue samples were then collected and their radioactivity was measured using a gamma counter (PerkinElmer, Waltham, MA). Data are expressed as the percentage of the injected dose per gram of tissue (% ID/g).

Results and Discussion

Purity of folic acid determined by HPLC

Folic acid was separated on an XBridge RP18 column (150 × 4.6 mm, 5 μm), using a mobile phase comprised of water (0.1% TFA):acetonitrile (0.1% TFA) under gradient conditions with a flow rate of 1.0 mL/min. Folic acid was detected at a wavelength of 290 nm. Under this condition, the retention time of folic acid was 16.7 min. The purity of the compound (96.5%) was
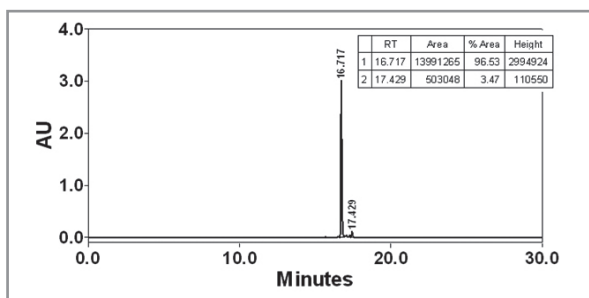
Figure 2. UV/HPLC profile folic acid separated using C18 column.

consistent with the purity stated by the manufacturer (Fig. 2).

Optimization of the ^{131}I-radiolabeling reaction using the iodobead method and iodic acid (HIO₃) method

In the chemical oxidation method, Na[131]I can be converted to its corresponding reactive radioactive iodine form. Robert et al. used iodogen as a mild oxidizing agent for radiolabeling the tyrosine group of folic acid with radioactive iodine, which did not affect biological activity of folic acid (10). In our experiment, we tested stronger oxidizing agents to determine the possibility of directly radiolabeling folic acid. In procedure 1, when we used the iodobead method, the radiolabeling reaction of folic acid did not occur at 30 °C or even at 60 °C during 1 h. There was no radioactivity peak that matched with the UV peak of folic acid (Fig. 3).

Next, we tested iodic acid as a stronger oxidizing agent. Folic acid (22.8 μg, 10 μL) was added to 100 μL of PBS, mixed with Na[131]I (3.7MBq) and HIO₃ (10 μL, 0.1 M), and the mixture shaken at 850 rpm for 1 h at 30 or 60 °C. The reaction did not proceed at 30 °C. When we increased the reaction temperature to 60 °C, two peaks were observed in the radio-HPLC chromatogram, with retention times of 18.4 and 19.3
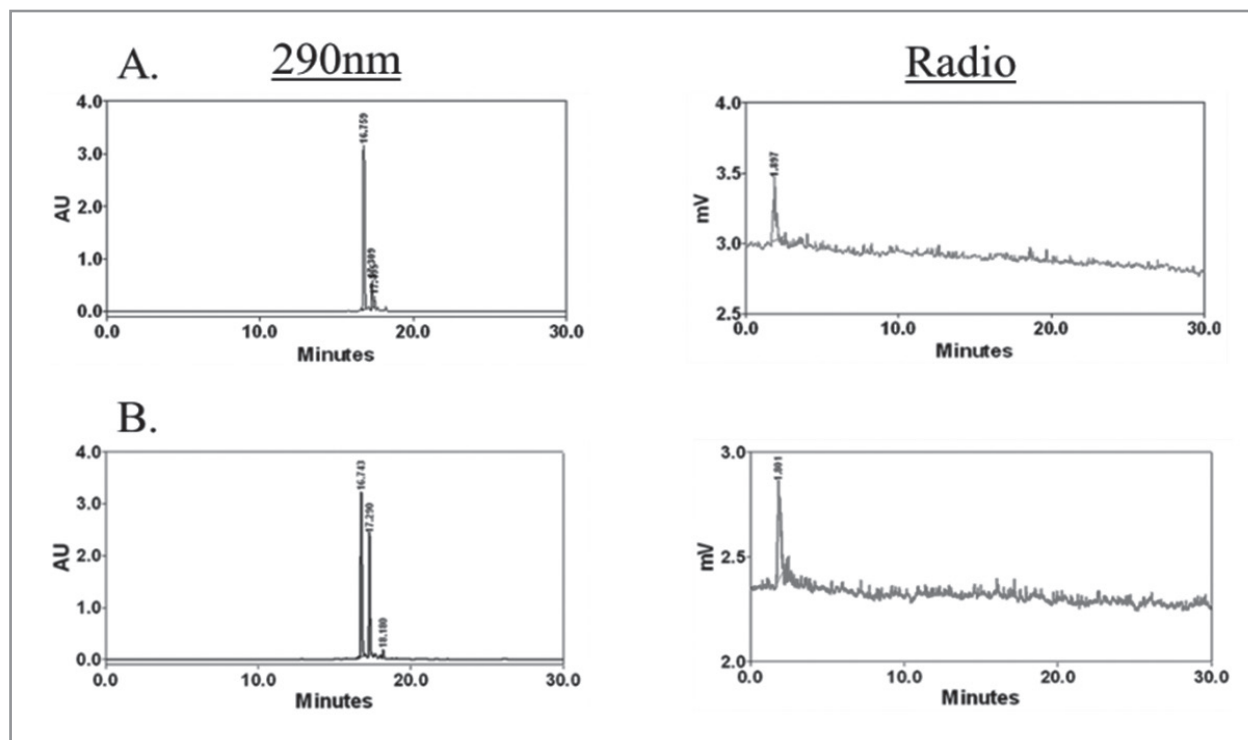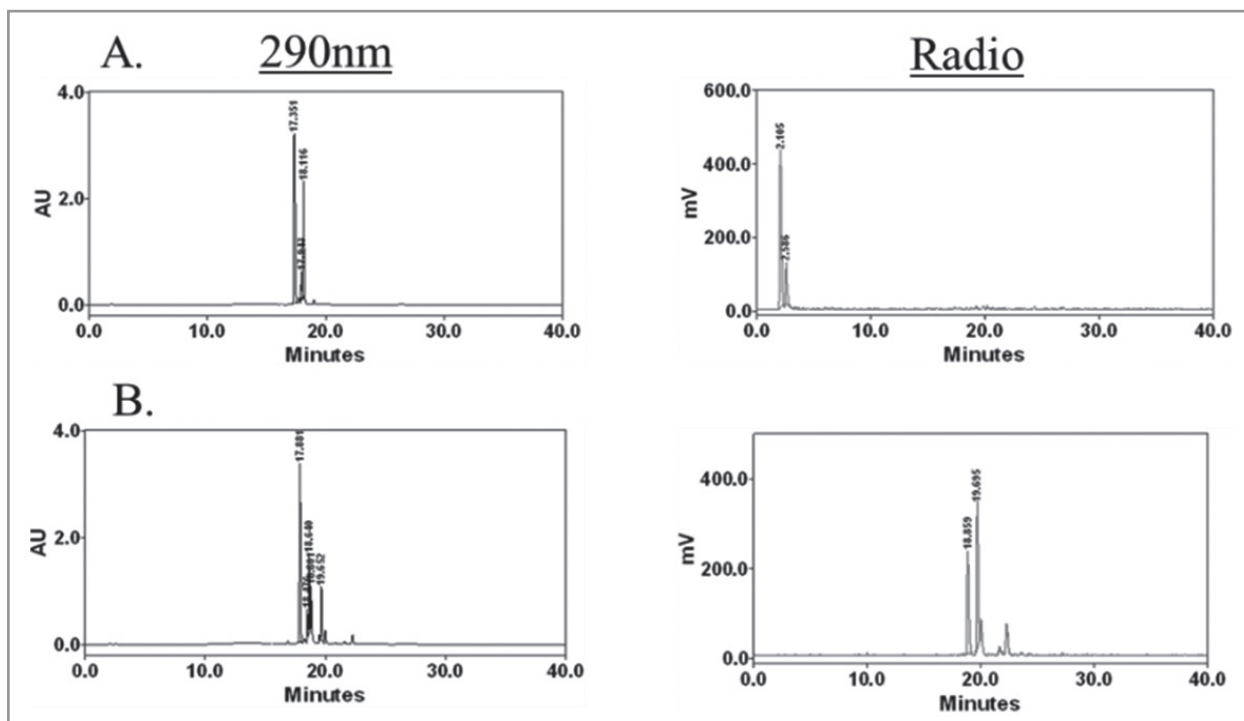

Figure 3. UV/radio-HPLC chromatograms of ^{131}I-radiolabeling with folic acid at 30 °C (A) and 60 °C (B), using the Iodobead® method.

Figure 4. UV/radio-HPLC chromatograms of ^{131}I-radiolabeling with folic acid at 30 °C (A) and 60 °C (B), using HIO₃.

min, respectively (Fig. 4).

So far, using 10 μL of HIO₃ (0.1 M) at 60 °C with an incubation time of 1 h was the best condition for radiolabeling folic acid with [131]I. Two radiolabeling compounds were obtained, presumably representing mono- and di-iodinated compounds. On the basis of their structures, we assumed that the peak at 18 min was mono-iodofolic acid and the peak at 19 min was di-iodofolic acid. The two radiolabeled compounds were separately collected by HPLC and confirmed by radio-TLC (Fig. 5).

Stability of ^{131}I-folic acid in PBS and human serum

After successfully radiolabeling folic acid with ^{131}I by the reaction of the compound with HIO₃ (0.1 M, 10 μL) at 60 °C for 1 h, we tested the stability of the radiolabeled compounds for further in vivo experiments.

First, the radiolabeled folic acid was purified by HPLC and divided into two samples. Each sample, with an activity of 100 μCi, was mixed with 500 μL of either PBS or human serum and incubated for various time periods (0–24 h) at 37 °C, with shaking at 750 rpm. The results showed that radioiodinated folic acid compound 1 was stable in PBS but not in human serum after 4 h of incubation. In contrast, the radioiodinated folic acid compound 2 showed good stability in both PBS and human serum for at least 24 h (Fig. 6).

Biodistribution experiments in a murine 4T1 tumor model

In vivo biodistribution studies were performed in 9-week-old female BALB/c mice (n = 1 per group) at the 1 and 4 h post injection. At the earlier time point (1 h), the highest radioactive signals were observed in the kidneys and liver with both radiolabeled compounds 1 and 2. At 4 h after injection, compound 1
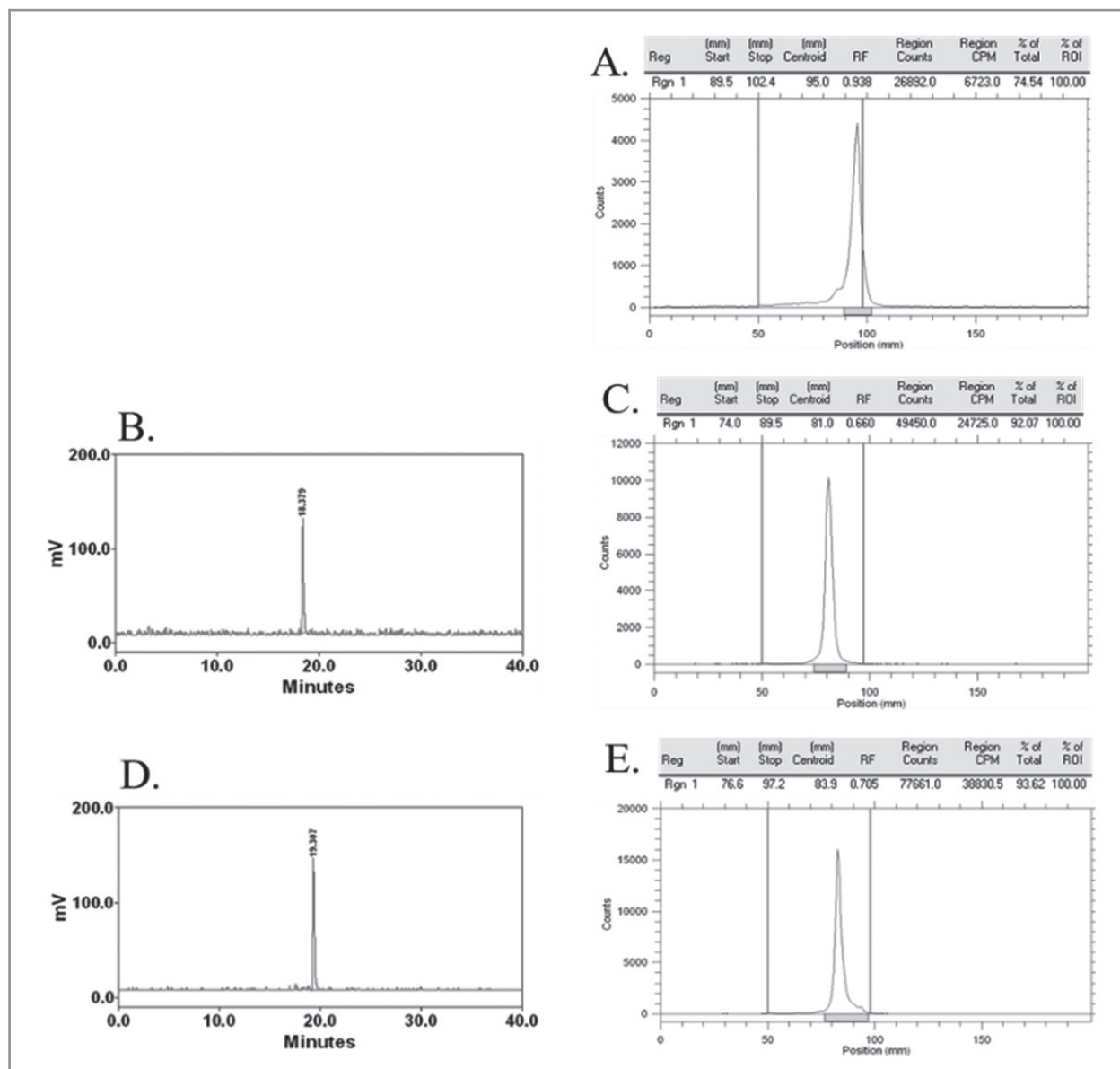
Figure 5. Radio-HPLC chromatograms and thin-layer chromatography (TLC) of purified ^{131}I-folic acid compounds. TLC of free ^{131}I ions (A), (B, C) radio-HPLC and TLC of purified ^{131}I-folic acid compound 1, (D, E) radio-HPLC and TLC of isolated ^{131}I-folic acid compound 2.
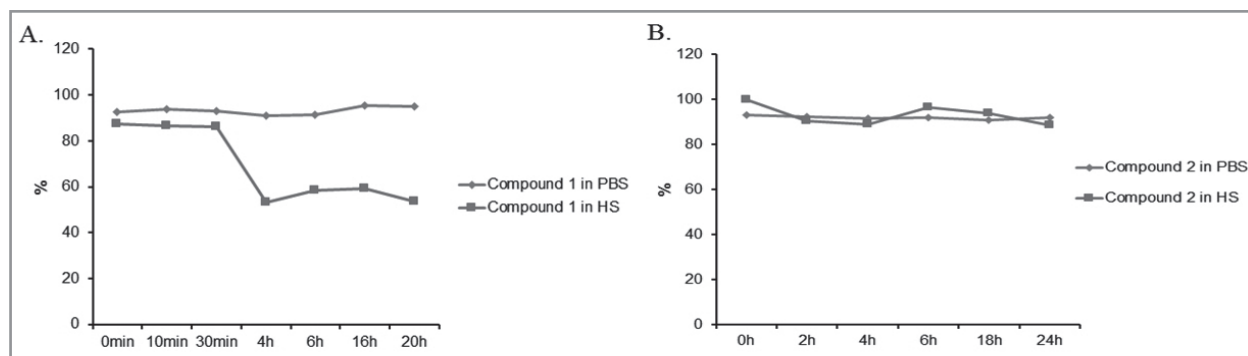

Figure 6. Stability of ^{131}I-folic acid compound 1 (A) and 2 (B) in PBS and human serum.

showed the highest uptake in the thyroid and stomach, which indicated a high rate of de-iodination from the radioiodinated compound 1 (Fig. 7a). This result was in good agreement with the in vitro stability data (Fig. 6a). Compound 2 showed a promising level of stability with much less de-iodination. However, there was no significant uptake into the tumor. At the 4-h time point, the highest uptake of the compound was detected in the kidneys and liver, which indicated that ^{131}I-folic acid compound 2 was rapidly cleared through both renal and hepatobiliary excretion (Fig. 7b). In reference 10, at early time points (1 and 4 h), a high uptake of radioactivity was also found in the thyroid gland because of accumulation of de-iodinated free ^{125}I ions. To decrease the uptake of radioactivity into the kidneys, the anti-folate drug pemetrexed was preinjected.

In future studies, we may apply other radiolabeling methods to develop a labeled folate compound with a higher tumor-specific uptake. According to Soroka (11) and Kulkarni (12), folic acid could be radiolabeled with ^{125}I using chloramine T, and they reported that a mono-iodinated compound (3′-iodofolic acid) was obtained. 3′-Iodofolic acid showed a high uptake in tumor (22 ± 2%), thyroid gland (23 ± 2), kidney (52 ± 5%),

and liver (27 ± 2%) tissues at 24 h. The radiolabeled compounds synthesized using HIO_3 in our study seem to be different from the reported compound obtained by the chloramine T method. Other tumor models such as the KB tumor and C6 glioma cell lines can be also used for directly comparing the two folic acid radiolabeling methods using HIO_3 and chloramine T.

Conclusion

Radiolabeling of folic acid with ^{131}I using iodic acidwas completed within 1 h at 60 °C. Two radiolabeled compounds were observed. The radiolabeled ^{131}I-folic acid compound 1 was unstable after 4 h in human serum. In contrast, the radiolabeled ^{131}I-folic acid compound 2 showed a high stability for at least 24 h in human serum. Biodistribution studies in 4T1 tumor model mice showed the highest uptake of radioiodinated folic acid compound 1 in the thyroid as a result of deiodination. The radioiodinated folic acid compound 2 showed reduced de-iodination. However, neither compound showed any specific uptake by the tumor.
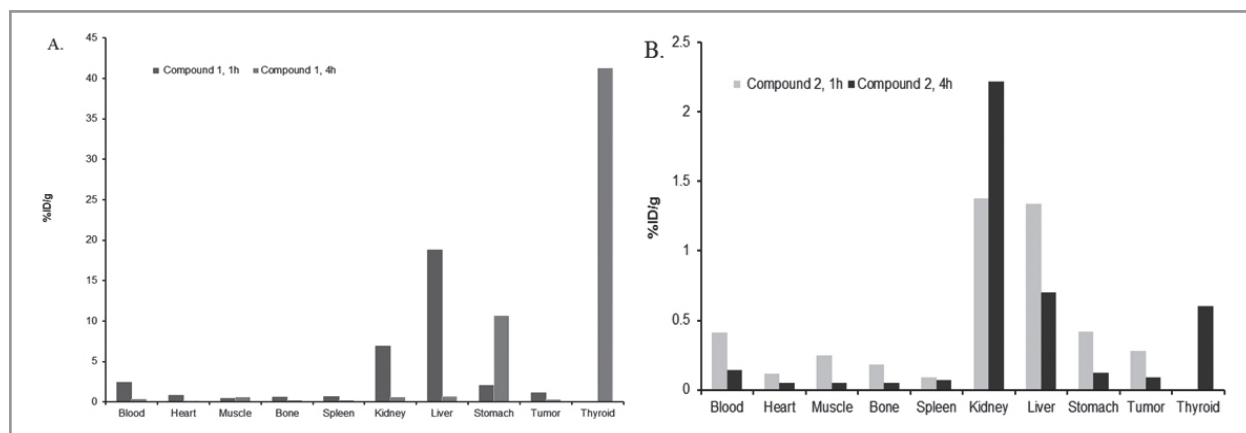
Figure 7. Biodistribution of ^{131}I-folic acid compounds 1 (A) and 2 (B) in female BALB/c mice 1 and 4 h post-injection.

Acknowledgment.

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