JPP Journal of Pharmacy and Pharmacology

JPP 2002, 54: 1073–1081

Received February 20, 2002
Accepted May 9, 2002
ISSN 0022-3573

Synthesis and evaluation of a monoreactive DOTA derivative for indium-111-based residualizing label to estimate protein pharmacokinetics

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Abstract

The purpose of this study was to develop an indium-111 (^{111}In)-based residualizing label for estimating the pharmacokinetics of proteins. 1,4,7,10-Tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (DOTA), which produced a highly stable and hydrophilic ^{111}In chelate, was selected as the chelating site, and the monoreactive DOTA derivative with a tetrafluorophenyl group as the protein binding site (mDOTA) was designed to avoid cross-linkings of proteins. mDOTA was synthesized with an overall yield of 11%. The stability in murine plasma, the radioactivity retention in the catabolic sites of proteins and the radiochemical yields of ^{111}In-labelled proteins via mDOTA were investigated using human serum albumin (HSA), galactosyl-neoglycoalbumin (NGA) and cytochrome c (cyt c) as model proteins. ^{111}In-labelled HSA via mDOTA was highly stable for 5 days after incubation in murine plasma. Long retention of radioactivity in the catabolic sites was observed after injection of ^{111}In-DOTA-NGA in mice, due to the slow elimination of the radiometabolite from the lysosome. At a chelator concentration of 42.2 μM, ^{111}In-DOTA-cyt c was produced with over 91% radiochemical yield. On the other hand, ^{111}In-DOTA-lysine and ^{111}In-DOTA were obtained with high radiochemical yields at lower chelator concentrations. These findings indicated that mDOTA would be an appropriate ^{111}In-labelling agent for estimating protein pharmacokinetics. These findings also suggested that the introduction of a protein binding site at a position distal from the unmodified DOTA structure would be preferable to preparing ^{111}In-DOTA-labelled proteins with higher specific activity.

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Acknowledgement: We are grateful to Nihon Medi-Physics Co. Ltd, Takarazuka, Japan for its kind gift of $^{111}InCl_3$.

Introduction

To assess the pharmaceutical applications of newly developed or chemically modified proteins, their in-vivo fate has to be investigated and evaluated quantitatively. Radiolabelling techniques have been used widely for these purposes because of their high sensitivity. In addition, single-photon emission computed tomography (SPECT) has become available to pursue the real-time localization of the radioactivity in living small laboratory animals (Weber et al 1994; Ishizu et al 1995). This procedure enables a real-time trace of the in-vivo behaviour of proteins in an animal by external measurement of the radioactivity. For the accurate estimation of protein pharmacokinetics, long residence times of the radioactivity at the catabolic sites are required. ^{14}C or ^{125}I labels with saccharide units have been developed for these purposes due to the generation of radiometabolites with limited ability to diffuse through the lysosomal membrane (Pittman et al 1979; Thorpe et al 1993). These radiolabelling reagents are called residualizing labels.

Recently, a metallic radionuclide, indium-111 (^{111}In) was used as a residualizing label after the reaction of proteins with bifunctional chelating agents (BCAs) (Mukai et al 1999a; Nishikawa et al 1999). ^{111}In emits gamma rays of adequate energies (173 and 247 keV) suitable for both radioactivity measuring with a gamma counter and external imaging using SPECT for small animals. The physical half-life (2.8 days) of this radionuclide is sufficient for pursuing the pharmacokinetics of proteins with a long plasma half-life but not too long for the disposal of radioactive materials. In addition,

^{111}In-labelled proteins are prepared with high radiochemical yields by a simple addition of ^{111}In solution to the conjugate solution, because of the rapid and high yield complexation reaction between chelator-conjugated proteins and ^{111}In (Hnatowich et al 1983). Cyclic diethylenetriaminepentaacetic anhydride (cDTPA) is used as the BCA to label proteins with ^{111}In, due to its simple conjugation reaction with proteins and its ready availability from commercial sources. Previous studies suggested that ^{111}In-labelled proteins via cDTPA exhibited relatively long residence of the radioactivity in the catabolic sites (Mukai et al 1999a; Nishikawa et al 1999). However, formation of inter- and intramolecular cross-linkings is unavoidable during the conjugation reaction since cDTPA possesses two binding sites for proteins (Maisano et al 1992; Reilly et al 1992; Arano et al 1996), which cause gradual release of ^{111}In from DTPA-conjugated proteins to transferrin while circulating in plasma (Arano et al 1996).

The purpose of this study was to develop a new ^{111}In-labelling agent suitable for estimating protein pharmacokinetics. 1,4,7,10-Tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (DOTA), which produced a highly stable and hydrophilic ^{111}In chelate (Wu et al 1992), was selected as the chelating site, and the monoreactive DOTA derivative with a tetrafluorophenyl group as the protein binding site (mDOTA) was designed and synthesized to avoid cross-linking of proteins. The plasma stability, radioactivity retention in the catabolic site and radiochemical yields of ^{111}In-DOTA-proteins were investigated using human serum albumin (HSA), galactosyl-neoglycoalbumin (NGA) and cytochrome c (cyt c), respectively, as model proteins. For estimation of plasma stability of ^{111}In-labelled proteins via mDOTA, the parental proteins should be stable in plasma so as not to generate any radiometabolites derived from parental proteins. Thus, we used a typical serum protein, HSA, as a model. NGA is a useful polypeptide to estimate the behaviour of radiometabolites generated after lysosomal proteolysis in hepatocytes (Arano et al 1994a, 1995; Mukai et al 1999a, b), and so this protein was used for evaluation of the residence time of the radioactivity derived from ^{111}In-DOTA labels in the catabolic site. In addition, the number of chelators attached per molecule of protein plays a critical role in radiochemical yields with ^{111}In. Since the exact number of chelators attached to cyt c was assessed by mass spectrometry (Lewis et al 1994), this protein was used as the model protein to evaluate the radiochemical yield of ^{111}In-DOTA-labelled proteins. The ability of mDOTA as the ^{111}In-labelling agent to estimate the protein pharmacokinetics was evaluated in comparison with cDTPA.

Materials and Methods

Materials

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker AC-200 spectrometer, and the chemical shifts were reported in ppm downfield from an internal tetramethylsilane standard. Fast atom bombardment mass spectra (FAB-MS) were obtained with a JMS-HX/HX 110 A model (JEOL Ltd, Tokyo, Japan). Size-exclusion HPLC (SE-HPLC) was performed using a 5 Diol-300 column (7.5 × 600 mm, Nacalai Tesque Inc., Kyoto, Japan), eluted with 0.1 M phosphate buffer (pH 6.8) at a flow rate of 1.0 mL min^{-1}. Cellulose acetate electrophoresis (CAE) strips were run in veronal buffer (pH 8.6, I = 0.06) at a constant current of 0.8 mA for 40 min. TLC analyses were performed with silica plates (Merck Art 5553) with 10% aqueous ammonium acetate–methanol (3:1) as the developing solvent. $^{111}InCl_3$ (74 MBq mL^{-1}) was supplied by Nihon Medi-Physics (Takarazuka, Japan). HSA and cyt c were purchased from Sigma Chemical Co. (St Louis, MO). NGA (44 galactoses per HSA) was prepared by the method reported previously (Mukai et al 1999b). cDTPA was obtained from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were of reagent grade.

Synthesis of mDOTA

*1,4,7,10-Tetraazacyclododecane-*N,N′,N″*-triacetic acid tri-*tert*-butyl ester (**1**)*

To Cyclem (1.66 g, 9.64 mmol) (Figure 1) dissolved in acetonitrile was added *N*,*N*-diisopropylethylamine (5.04 mL,28.9 mmol)and*tert*-butylbromoacetate(4.24 mL, 28.9 mmol), successively, while keeping the reaction temperature below 0°C. After 13 h stirring at room temperature, the solution was concentrated in-vacuo. Chloroform was added to the residue, and the organic phase was washed with saturated aqueous NaCl. After drying the organic phase over $CaSO_4$, the solution was evaporated and the residue was chromatographed on silica gel ($CHCl_3$–*tert*-BuOH 5:1) to afford **1** (2.02 g, 40.8%). ^{1}H NMR ($CDCl_3$): δ 1.41–1.53 (27H, s), 2.85–2.95 (12H, t), 3.07–3.14 (4H, t), 3.27–3.29 (2H, s), 3.36–3.39 (4H, s). FAB-MS calcd for $C_{26}H_{50}N_4O_6$ (M+H): m/z 515, found 515. Anal. calcd for ($C_{26}H_{50}N_4O_6 \cdot 3/5CHCl_3$) C, H, N.

N-*(Carbobenzoxymethyl)-1,4,7,10-tetraazacyclododecane-*N′,N″,N‴*-triacetic acid tri-*tert*-butyl ester (**2**)*

To a suspension of 60% NaH (0.121 g, 2.38 mmol) in dry dimethylformamide (DMF), was added compound **1** (1.08 g, 1.58 mmol) in dry DMF to maintain the reaction temperature below −15°C. After 2 h stirring at room temperature, benzyl bromoacetate (1.24 mL, 6.33 mmol) was added drop-wise below 0°C, and the reaction mixture was stirred for 27 h at room temperature. A 20-mL solution of 5% citric acid was then added to the reaction mixture before extraction with chloroform (3 × 20 mL). The organic phase was dried and evaporated, and the residue was chromatographed on silica gel ($CHCl_3$–*tert*-BuOH 5:1) to yield **2** (0.597 g, 42.8%). ^{1}H NMR ($CDCl_3$): δ 1.24–1.49 (27H, m), 2.15–3.54 (24H, m), 5.13 (2H, d), 7.33–7.42 (5H, m). FAB-MS calcd for $C_{35}H_{58}N_4O_8$ (M+Na): m/z 685, found 685.

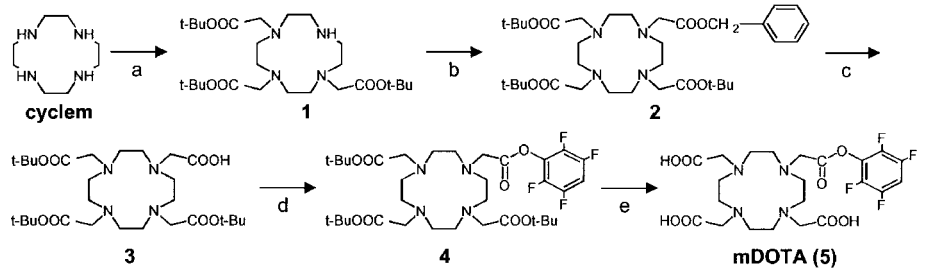

Figure 1 Synthesis of mDOTA. Reagents: a, *tert*-butyl bromoacetate, *N*,*N*-diisopropylethylamine; b, benzyl bromoacetate, NaH; c, H_2, Pd/C; d, 2,3,5,6-tetrafluorophenol, 1,3-dicyclohexylcarbodiimide; e, trifluoroacetic acid, anisole.

N-*(Carboxymethyl)-1,4,7,10-tetraazacyclododecane-*N′,N″,N‴*-triacetic acid tri*-tert-*butyl ester (DOTA(*tert-*Bu)*$_3$ *(**3**)*

A mixture of compound **2** (0.597 g, 0.901 mmol) and 10% palladium on carbon (0.4 g) in methanol was hydrogenated at room temperature for 3 h. The catalyst was removed by filtration, and the filtrate was evaporated to produce **3** (0.439 g, 85.1%). ^{1}H NMR ($CDCl_3$): δ 1.46–1.47 (27H, m), 1.91–3.65 (24H, m). FAB-MS calcd for $C_{28}H_{52}N_4O_8$ (M+Na): m/z 595, found 595.

N-*[Carbo(2,3,5,6-tetrafluorophenoxyl)methyl]-1,4,7,10-tetraazacyclododecane-*N′,N″,N‴*-triacetic acid tri*-tert-*butyl ester (**4**)*

To a mixture of compound **3** (0.100 g, 0.175 mmol) and 2,3,5,6-tetrafluorophenol (29.0 mg, 0.175 mmol) in dry chloroform, was added 1,3-dicyclohexylcarbodiimide (36.0 mg, 0.175 mmol) in dry chloroform to maintain the reaction temperature below 0°C. After 9 h stirring at room temperature, the reaction mixture was filtered, and the filtrate was concentrated in-vacuo. The residue was chromatographed on silica gel ($CHCl_3$–MeOH 16:1) to produce **4** (0.117 g, 93.0%). ^{1}H-NMR ($CDCl_3$): δ 1.24–1.46 (27H, m), 2.16–3.98 (24H, m), 7.08–7.19 (1H, m). FAB-MS calcd for $C_{34}H_{52}N_4O_8F_4$ (M+Na): m/z 743, found 743. Anal. calcd for ($C_{34}H_{52}N_4O_8F_4 \cdot 5/2CHCl_3$) C, H, N.

N-*[Carbo(2,3,5,6-tetrafluorophenoxyl)methyl]-1,4,7,10-tetraazacyclododecane-*N′,N″,N‴*-triacetic acid (mDOTA, **5**)*

Compound **4** (0.104 g, 0.144 mmol) was stirred in a mixed solution of trifluoroacetic acid (2 mL) and anisole (0.1 mL) for 30 h at room temperature. After removal of trifluoroacetic acid in-vacuo, the residue was treated with dry ether to precipitate **5** (63.0 mg, 79.0%). ^{1}H NMR ($(CD_3)_2SO$): δ 2.98–3.80 (24H, m), 7.20–7.28 (1H, m). FAB-MS calcd for $C_{22}H_{28}N_4O_8F_4$ (M+Na): m/z 575, found 575.

Synthesis of DOTA-lysine

To a mixture of DOTA(*tert*-Bu)$_3$ (0.130 g, 0.227 mmol), N^{α}-*tert*-butoxycarbonyl-L-lysine methyl ester (Boc-Lys-OMe; 0.136 g, 0.681 mmol) and 1-hydroxybenzotriazole (92.0 mg, 0.681 mmol) in chloroform, was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.131 g, 0.681 mmol) in chloroform below 0°C. After 13 h stirring at room temperature, the reaction mixture was washed with 5% citric acid, dried and evaporated. The residue was chromatographed on silica gel ($CHCl_3$–MeOH 8:1) to produce DOTA(*tert*-Bu)$_3$-Boc-Lys-OMe (63.0 mg, 33.9%). ^{1}H NMR ($CDCl_3$): δ 1.25–1.78(36H+8H, m), 2.05–3.39 (24H, m), 3.72 (3H, s), 4.17–4.24 (1H, m), 5.19–5.25 (1H, br). FAB-MS calcd for $C_{40}H_{74}N_6O_{11}$ (M+Na): m/z 837, found 837.

DOTA(*tert*-Bu)$_3$-Boc-Lys-OMe (62.0 mg, 76.0 μmol) was dissolved in ethanol (2 mL), and 2 M NaOH (4 mL) was added. After 4 h stirring at room temperature, the solution was acidified to pH 3.5 with 4 M H_2SO_4 before extraction with chloroform. The organic phase was dried and evaporated. A mixed solution of trifluoroacetic acid and anisole was added to the residue, and the reaction solution was stirred for 30 h at room temperature. After removal of trifluoroacetic acid in-vacuo, dry ether was added to produce DOTA-lysine (22.0 mg, 53.7%). ^{1}H NMR (D_2O): δ 1.15–1.48 (6H, m), 1.75–1.82 (2H, m), 2.94–3.15 (16H, m), 3.62–3.81 (8H, m), 4.90–4.97 (1H, m), 5.31–5.36 (1H, br). FAB-MS calcd for $C_{22}H_{40}N_6O_9$ (M+H): m/z 533, found 533.

Conjugation of mDOTA with proteins

To a solution of HSA, NGA and cyt c (9 mg mL^{-1}) in 500 μL of borate-buffered saline (0.1 M, pH 9.0), 10, 30 and 20 molar excess of mDOTA in 5 μL of dimethyl sulfoxide (DMSO) was added, respectively. Previous studies suggested that DMSO at this concentration had no effect on the biological activity of proteins (Hartikka et al 1989; Nishikawa et al 1999). After incubating for 13 h at room temperature, the conjugate was purified by the centrifuged column procedure using Sephadex G-50 (Pharmacia Biotech, Tokyo, Japan), equilibrated with 0.5 M acetate buffer (pH 6.0). Conjugation of cDTPA with proteins was performed as described previously (Arano et al 1994a). The average number of DOTA or DTPA chelates incorporated into cyt c was found to be 2, as determined by FAB-MS.

^{111}In labelling of proteins

To 7 μL of acetate buffer (3 M, pH 6.0), was added 35 μL of $^{111}InCl_3$. After the mixture was allowed to stand for 5 min

at room temperature, a 26-μL solution of DOTA- or DTPA-conjugated proteins (3.0–10 mg mL^{-1}) was added. After incubating for 1.5 h at 37°C, the conjugate was purified by the centrifuged column procedure using Sephadex G-50, equilibrated and eluted with 0.1 M phosphate buffer (pH 7.4). The radiochemical purity of ^{111}In-labelled proteins was determined by SE-HPLC, CAE and TLC.

For ^{111}In labelling of bovine apotransferrin (Nacalai Tesque Inc.), a 40-μL solution of $^{111}InCl_3$ was mixed with a 40-μL solution of 1 M sodium acetate, and the resulting ^{111}In-acetate solution (50 μL) was added to an apotransferrin solution (150 μL; 5 mg mL^{-1}) in 0.1 M phosphate buffer (pH 7.4). After incubating for 10 min at 37°C, a 20-μL solution of 2 mM EDTA was added. After a 30-min incubation, ^{111}In-labelled transferrin was purified by the centrifuged gel-filtration column.

Plasma stability of ^{111}In-labelled HSA

^{111}In-DOTA-HSA and ^{111}In-DTPA-HSA were diluted 20 fold with freshly prepared murine plasma containing 0.1% sodium azide. After 1, 2, 3 and 5 days of incubation at 37°C, the samples were analysed by CAE. Since $^{111}In^{3+}$, released from ^{111}In-labelled proteins in the circulation, forms a strong complex with transferrin (a serum iron transport protein) (Himmelsbach & Wahl 1989; Reilly et al 1992; Claessens et al 1995), ^{111}In-labelled transferrin was used as an analytical reference.

Biodistribution of ^{111}In-labelled NGA

Animal studies were conducted in accordance with our institutional guidelines and were approved by Kyoto University Animal Care Committee. Biodistribution studies were performed by the intravenous administration of ^{111}In-labelled NGA (9 μg) to 6-week-old male ddY mice (27–30 g). At 10 and 30 min, 1, 3, 6 and 24 h post-injection, mice were killed by decapitation and samples of blood were collected. Liver, kidney, spleen, stomach, intestine and lung were excised and weighed, and the radioactivity counts were determined with an auto well gamma counter (ARC 2000, Aloka, Tokyo, Japan).

Identification of radiometabolites of ^{111}In-labelled NGA

To identify the radiometabolites, ^{111}In-DOTA-NGA was injected intravenously into mice. At 1 and 24 h post-injection, liver homogenate was prepared according to the method described previously (Arano et al 1994b). Supernatants were separated from the pellets, filtered through a polycarbonate membrane with a pore diameter of 0.45 μm, and analysed by SE-HPLC, CAE and TLC. Each analysis was also carried out by co-chromatography with ^{111}In-DOTA-lysine and ^{111}In-DOTA.

Subcellular distribution of radiometabolites in the liver

The subcellular distribution of radioactivity in the liver was investigated by density-gradient centrifugation. At 1 and 24 h post-injection of ^{111}In-DOTA-NGA, liver homogenate was prepared according to the method described previously (Arano et al 1994a; Mukai et al 1999b). The isolated supernatant was then layered on the isotonic Percoll (Pharmacia Biotech) at a density of 1.08 g mL^{-1}. After centrifugation at 20 000 g at 4°C for 90 min (RP 30 rotor Hitachi Co. Ltd, Tokyo, Japan), the gradients were collected in 14 fractions, and the activity of lysosomal enzyme (β-galactosidase), the density and radioactivity of respective fractions were analysed.

Radiochemical yields of ^{111}In-labelled cyt c

The chelator concentrations of DOTA- and DTPA-cyt c (2.0 each chelator per cyt c) were adjusted to 5.6, 16.9, 28.1, 42.2 and 84.4 μM with 0.5 M acetate buffer (pH 6.0). To 7 μL of acetate buffer (3 M, pH 6.0), was added 35 μL of $^{111}InCl_3$, and the solution was incubated for 5 min before adding to 26 μL of each chelator-conjugated cyt c. After incubating for 1.5 h at 37°C, EDTA was added to a 100 molar excess over each protein molecule, and the reaction mixture was gently agitated for 30 min at room temperature. The radiochemical yields of ^{111}In-labelled proteins were determined by CAE. DOTA-lysine and DOTA were labelled with ^{111}In in the same manner, and the radiochemical yields were determined by TLC.

Statistical analysis

Percentages of HSA-bound radioactivity after incubation of ^{111}In-labelled HSA in murine plasma were expressed as the mean$\pm$s.d. of three experiments, and the statistical significance of the difference between ^{111}In-DOTA-HSA and ^{111}In-DTPA-HSA was calculated using the Mann–Whitney U-test. Experimental results regarding mouse distribution of ^{111}In-labelled NGA were given as the mean$\pm$s.d. of five experiments, and the statistical analysis was performed by applying the Mann–Whitney U-test. ^{111}In-complexation yields of cyt c conjugates were expressed as the mean$\pm$s.d. of three experiments, and the statistical significance of the difference between ^{111}In-DOTA-cyt c and ^{111}In-DTPA-cyt c was estimated using the Mann–Whitney U-test. $P < 0.05$ was considered to be statistically significant.

Results and Discussion

In a previous study, Lewis et al (1994) synthesized the N-hydroxysulfosuccinimide ester of DOTA in the presence of water-soluble carbodiimide to prepare ^{111}In-labelled proteins. This procedure may be applicable to assess ^{111}In-DOTA as a residualizing label for protein pharmaco-

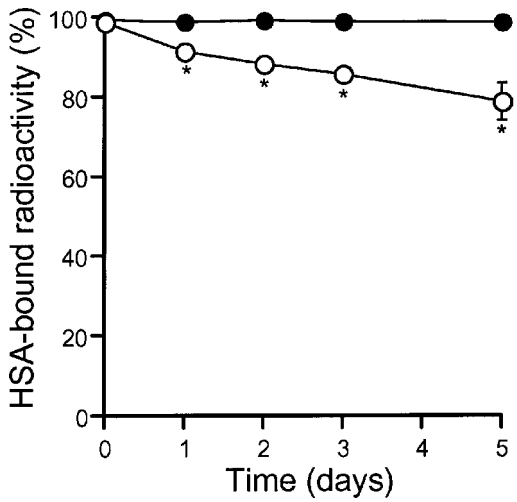

Figure 2 Stability of ^{111}In-labelled HSA after incubation in murine plasma at 37°C. ●, ^{111}In-DOTA-HSA; ○, ^{111}In-DTPA-HSA. Each value represents the mean±s.d. of three experiments. $*P < 0.05$ compared with ^{111}In-DOTA-HSA (Mann–Whitney *U*-test).

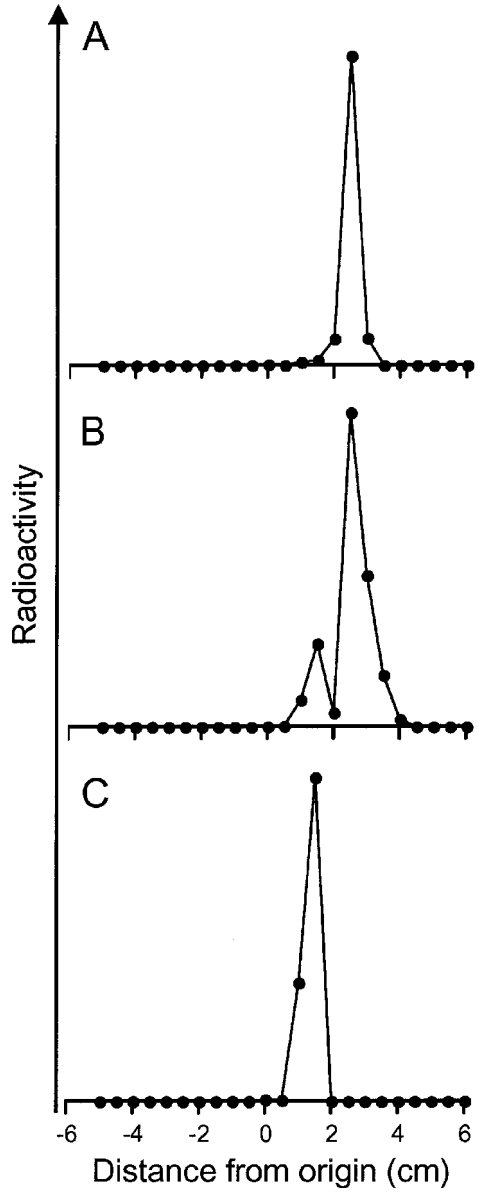

Figure 3 Cellulose acetate electrophoresis (CAE) profiles at 5 days after incubation of ^{111}In-DOTA-HSA (A) and ^{111}In-DTPA-HSA (B) in murine plasma at 37°C. The peak of ^{111}In-labelled apotransferrin was observed at 0.5–1 cm anode from the origin (C).

kinetics. However, since the active ester was not isolated from the reaction solution before conjugation reactions with proteins, a small amount of multiple activation of DOTA was observed in the preparations. When the DOTA-antibody conjugates were prepared by using the *N*-hydroxysulfosuccinimide ester, approximately 6% of the antibody was converted to dimers (Lewis et al 1994). The intermolecular cross-linked species such as antibody dimers could be removed from the protein conjugates during purification, as they described. However, the products of intramolecular cross-linking were difficult to detect and remove, and would impair the ^{111}In-chelate stability, as was observed in cDTPA radiolabelling of proteins (Arano et al 1996). To estimate the ability of DOTA as an ^{111}In-based residualizing label of proteins, we developed a synthetic procedure for a monoreactive DOTA derivative to avoid cross-linking of proteins.

Monoreactive DOTA was prepared by protecting one carboxylate with a reagent that can be removed under conditions different from the protecting groups of the other three carboxylates in the DOTA skeleton. We selected the benzyl-protecting group for one carboxylate and the *tert*-butyl-protecting group for the rest of the three carboxylates (compound **2**). The precursor of the monoreactive DOTA, compound **3,** was produced in relatively high yield (85.1%) by catalytic hydrogenation of compound **2**. This compound is a monocarboxylic acid derivative of DOTA with the remainder of the three carboxylates being protected with acid-labile *tert*-butyl ester. The high solubility of this compound in a variety of organic solvents would render the reagent versatile for introducing a DOTA skeleton to a variety of peptides by either liquid or solid synthesis for diagnostic and therapeutic applications in nuclear medicine.

We attempted to convert the free carboxylate of compound **3** to *N*-hydroxysuccinimide ester and subsequent deprotection of the *tert*-butyl esters. However, *N*-hydroxysuccinimide ester underwent decomposition during the deprotection reaction. Therefore, we selected a tetrafluorophenyl ester as a protein binding site. According to the procedure outlined in Figure 1, mDOTA was synthesized with an overall yield of 11%, and the chemical purity of mDOTA synthesized was found to be over 99%, as determined by TLC.

The stability of ^{111}In-labelled proteins in murine plasma was investigated using HSA as a model protein (Figure 2). Figures 3A and 3B show typical CAE profiles at 5 days after incubation of ^{111}In-DOTA-HSA and ^{111}In-DTPA-

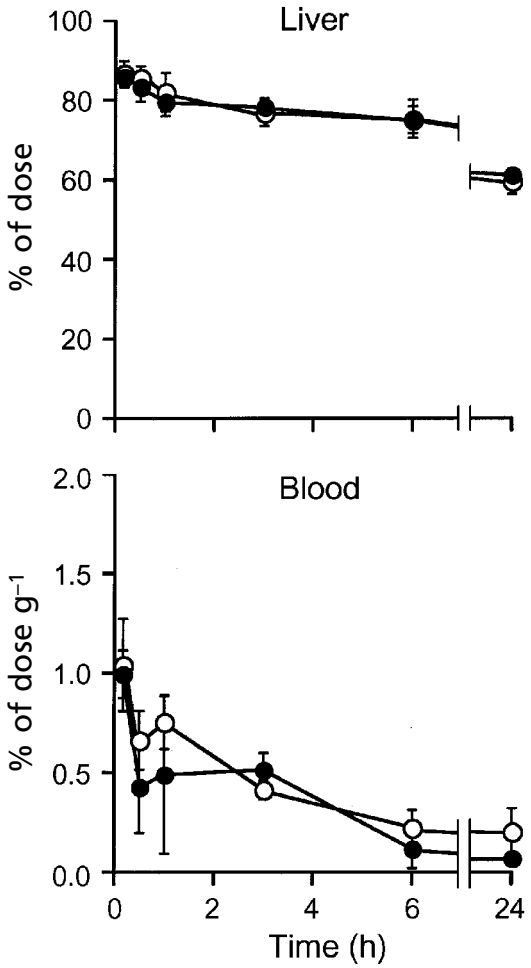

Figure 4 Radioactivity profiles in the liver and blood after injection of ^{111}In-labelled NGA into mice. ●, ^{111}In-DOTA-NGA; ○, ^{111}In-DTPA-NGA. Each value represents the mean±s.d. of five experiments. No significant difference between ^{111}In-DOTA-NGA and ^{111}In-DTPA-NGA was seen in the hepatic and blood radioactivity levels at any post-injection time (Mann–Whitney *U*-test).

HSA, respectively. The CAE profile of ^{111}In-labelled apotransferrin is also illustrated in Figure 3C. The radioactivity associated with HSA fractions of ^{111}In-DOTA-HSA was unchanged for 5 days, indicating high stability of ^{111}In-DOTA labels. For the assessment of protein pharmacokinetics, high stability of radiolabels is required. The ^{111}In-labelling procedure using mDOTA would satisfy this requirement although one of the four carboxylates in the DOTA molecule was utilized for protein conjugation. On the other hand, ^{111}In-DTPA-HSA released the radioactivity, and approximately 20% of the initial radioactivity was detected in the transferrin fraction at 5 days. These observations reconfirmed the involvement of transchelation of ^{111}In from ^{111}In-DTPA-labelled proteins to transferrin in plasma (Himmelsbach & Wahl 1989; Reilly et al 1992; Claessens et al 1995). The low stability of ^{111}In-DTPA-labelled proteins is not attributable to low stability of ^{111}In-DTPA chelate, but to formation of intramolecular cross-linking during cDTPA conjugation reactions (Arano et al 1996). Therefore, the ^{111}In-labelling method using cDTPA would be unsuitable for pursuing the pharmacokinetics of proteins with long biological half-lives of a few days.

To assess the protein pharmacokinetics accurately, long retention of the radiometabolites at the catabolic sites is required (Thorpe et al 1993). Our previous findings suggested that the ^{111}In-DTPA-labelled proteins, without inducing cross-linking, exhibited long residence of the radioactivity in the catabolic sites (Arano et al 1994a; Mukai et al 1999a). For further evaluation of the ^{111}In-DOTA label as a residualizing label, the residence time of the radioactivity in the catabolic site was investigated after administration of ^{111}In-DOTA-labelled proteins and compared with ^{111}In-DTPA-labelled proteins using NGA as a model protein. Use of NGA provides explicit information regarding the fate of radioactivity after lysosomal proteolysis in hepatocytes without being affected by transchelation of radiolabels in plasma or a redistribution of radiometabolites generated elsewhere in the body to the liver (Arano et al 1994a, 1995; Mukai et al 1999a, 1999b). Figure 4 shows the radioactivity profiles in the liver and blood after injection of ^{111}In-DOTA-NGA and ^{111}In-DTPA-NGA into mice. Approximately 86% of the injected radioactivity accumulated in the liver at 10 min post-injection of ^{111}In-DOTA-NGA, while only low radioactivity existed in the blood at this post-injection time (Figure 4). Elimination of radioactivity from the liver was slow, with 61% of the injected radioactivity remaining even at 24 h post-injection. No significant difference was seen in the hepatic radioactivity levels at any post-injection times between ^{111}In-DOTA-NGA and ^{111}In-DTPA-NGA. Low radioactivity, amounting to less than 4.0% of that injected, was detected in kidney, spleen, stomach, intestine and lung after injection of ^{111}In-labelled NGA (data not shown). The high plasma stability and long retention of radioactivity at the catabolic sites indicated that the ^{111}In-labelling procedure using mDOTA as a BCA would be applicable for estimating protein pharmacokinetics.

To determine the radiometabolites trapped in the catabolic site, analyses of the supernatant of the liver homogenate at 1 and 24 h post-injection of ^{111}In-DOTA-NGA were performed (Figure 5). Extraction efficiency of the radioactivity from each homogenate was more than 93%. On the basis of the previous findings that ^{111}In-DTPA-NGA was degraded to ^{111}In-DTPA-lysine in the liver (Arano et al 1994c; Franano et al 1994), we synthesized ^{111}In-DOTA-lysine as an authentic standard. The SE-HPLC profile of each supernatant depicted a single radioactivity peak at 28.5 min, a retention time that was representative of small-molecular-weight compounds such as ^{111}In-DOTA-lysine and ^{111}In-DOTA. Under similar conditions, ^{111}In-DOTA-NGA was eluted at 17 min. CAE analyses showed the major radioactivity peak at 0.5–1 cm cathode from the origin, which was identical to ^{111}In-DOTA-lysine (Figure 5B). TLC analyses registered the major radioactivity peak with an Rf value similar to that of ^{111}In-DOTA-lysine (Figure 5C). In every analytical system, each sample showed a single radioactivity peak even when

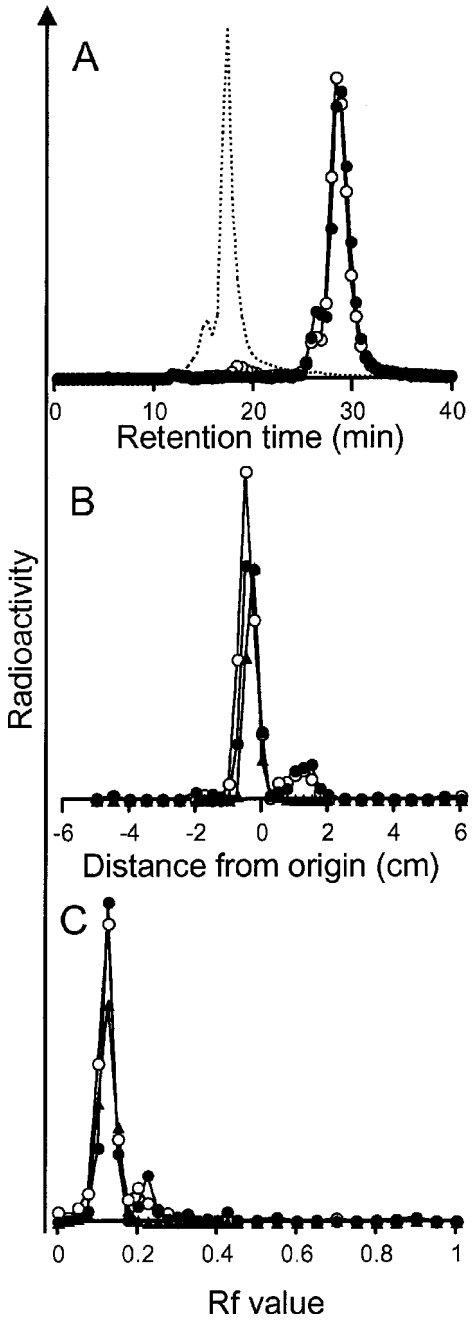

Figure 5 Chromatographic analyses of liver homogenates after injection of ^{111}In-DOTA-NGA into mice. ●, liver homogenate at 1 h; ○, liver homogenate at 24 h; ▲, ^{111}In-DOTA-lysine. Supernatants were analysed by SE-HPLC (A), CAE (B) and TLC (C). The parental ^{111}In-DOTA-NGA (dotted line) was eluted at 17 min on SE-HPLC (A). The peak of ^{111}In-DOTA was observed at 1 cm anode on CAE or Rf value of ca. 0.2 on TLC.

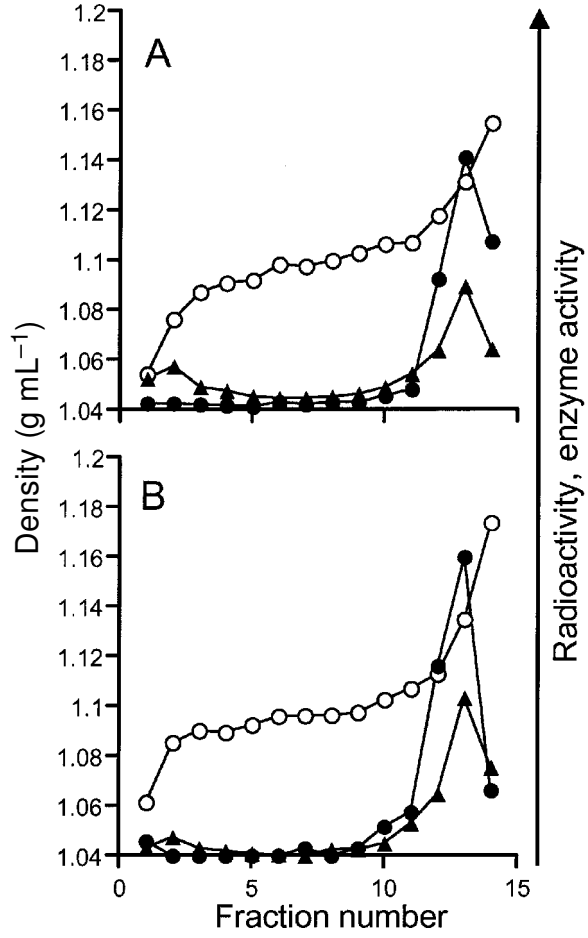

Figure 6 Percoll density gradient profiles of liver homogenate at 1 h (A) and 24 h (B) post-injection of ^{111}In-DOTA-NGA into mice. A single radioactivity peak (●) that coincided with β-galactosidase activity (▲) was detected at a density (○) of ca. 1.13 g mL^{-1}.

co-chromatographed with ^{111}In-DOTA-lysine (data not shown). These results are supported by the recent finding of Tsai and coworkers who demonstrated a generation of this radiometabolite in the kidney following administration of ^{111}In-DOTA-conjugated antibody fragments (Tsai et al 2001). These findings indicated that transchelation of ^{111}In from ^{111}In-DOTA chelate to biomolecules inside the cells was negligible.

The subcellular distribution of radioactivity in liver homogenates at 1 and 24 h post-injection of ^{111}In-DOTA-NGA was investigated by Percoll density gradient centrifugation (Figure 6). Each liver homogenate demonstrated a single radioactivity peak at a density of ca. 1.13 g mL^{-1}, which correlated well with the lysosomal enzyme (β-galactosidase) activity profile. Thus, the long residence times of the radioactivity in the catabolic site of ^{111}In-DOTA-labelled proteins would be attributable to the slow elimination rate of the final radiometabolite, ^{111}In-DOTA-lysine, from the lysosomal compartment.

To pursue the real-time localization of the radioactivity in small animals by SPECT, a high dose of radioactivity has to be administered into the animals. Thus, cyt c was used as a model protein to evaluate the radiochemical yield of the ^{111}In-DOTA-labelled proteins, since the number of chelators introduced per molecule of cyt c was assessed by mass spectrometry (Lewis et al 1994). ^{111}In-complexation

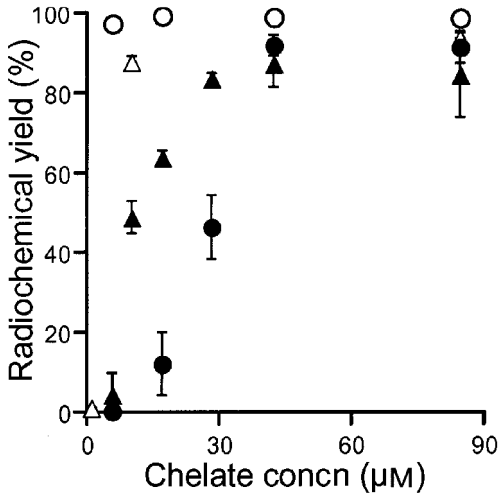

Figure 7 ^{111}In-complexation yields of chelator-conjugates. ●, DOTA-cyt c; ○, DTPA-cyt c; ▲, DOTA-lysine; △, DOTA. Radiolabelling was performed by incubating chelator conjugates with ^{111}In-acetate complexes for 1.5 h at 37°C. Each value represents the mean±s.d. of three experiments. The radiochemical yield of ^{111}In-DTPA-cyt c was significantly higher than that of ^{111}In-DOTA-cyt c at any concentration ($P < 0.05$, Mann–Whitney U-test).

yields of DOTA-cyt c and DTPA-cyt c (2.0 chelates per cyt c) are summarized in Figure 7. At chelator concentrations of 84.4 and 42.2 μM, DOTA-cyt c generated ^{111}In-labelled cyt c of 81.4 and 159 MBq mg^{-1} specific activity, respectively, with over 91% radiochemical yields. However, significant decreases in the radiochemical yields were observed with a decrease in the chelator concentrations. On the other hand, DTPA-cyt c produced ^{111}In-labelled cyt c of 1.20 GBq mg^{-1} specific activity, with over 97% radiochemical yields at lower chelator concentrations (5.62 μM). For further pursuit of the ^{111}In-complexation reactions of DOTA-conjugated proteins, DOTA-lysine and DOTA were also labelled with ^{111}In. ^{111}In-DOTA-lysine and ^{111}In-DOTA were obtained with over 84% radiochemical yields at chelator concentrations of more than 28.1 and 10.0 μM, respectively (Figure 7). These observations suggested that the relatively low specific activity of ^{111}In-DOTA-cyt c could be attributed not only to a steric interference of protein molecules but also to the modification of one carboxylate of DOTA (Keire & Kobayashi 1999).

Conclusions

We developed mDOTA as a BCA to label proteins with ^{111}In. ^{111}In-labelled proteins using mDOTA as a BCA were highly stable in-vitro and in-vivo. Furthermore, long residence times of the radioactivity in the catabolic site were observed after injection of ^{111}In-DOTA-labelled proteins, due to the slow elimination rate of the radiometabolite from the lysosome. Thus, mDOTA appears to be an appropriate ^{111}In-labelling agent for estimating protein pharmacokinetics. The findings of this study also suggested introduction of a protein binding site at a position distal from an intact DOTA structure would be preferable to prepare ^{111}In-labelled proteins of high specific activity for SPECT studies.

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