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Labeling Peptides with Technetium-99m Using a Bifunctional Chelator of a N-Hydroxysuccinimide Ester of Mercaptoacetyltriglycine

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A modified mercaptoacetyltriglycine (MAG3) chelator, which has acetyl S-protection and which is derivitized with N-hydroxysuccinimide (NHS) ester for conjugation, has been used to radiolabel four small (~6- to 7-kDa) peptides, bovine pancreatic trypsin inhibitor, epidermal growth factor, human neutrophil elastase inhibitor and plasmin inhibitor, with ^{99m}Tc. **Methods:** Each peptide was specifically labeled at the MAG3 chelation sites at ambient temperature and neutral pH. Specific activities of 100–150 mCi/mg were achieved at labeling efficiencies of about 50%, but specific activities of 3500 mCi/μmol could be attained. **Results:** By a variety of assays, protein activity was unimpaired by the conjugation and labeling for two of the four peptides. The activities for plasmin of the plasmin inhibitor and bovine pancreatic trypsin inhibitor were reduced by conjugation, presumably because of a sensitive lysine residue in the structure of each of these two peptides. Multiple peaks were present in the high-performance liquid chromatography radiochromatograms, especially of human neutrophil elastase inhibitor; however, most peaks could be shown to be labeled active peptide. Stability during cysteine challenge at modest cysteine-to-peptide molar ratios and during incubation in serum was observed in each case. Large differences among the labeled peptides were apparent in the 3-hr biodistributions of ^{99m}Tc in normal mice. **Conclusion:** The use of NHS-S-acetyl-MAG3 may be a convenient method of radiolabeling peptides with ^{99m}Tc.

Key Words: peptides; MAG3; technetium-99m

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The development of labeled small peptides as radiopharmaceuticals has lately become a minor growth industry, driven, in part, by the success of radiolabeled octeotride in localizing somatostatin receptors (*1*). The list of peptides currently being investigated as potential radiopharmaceuticals for imaging is long (*2*) and growing (*3,4*).

Peptides have been radiolabeled with the radioisotopes of iodine (^{123}I, ^{125}I and ^{131}I), ^{111}In, ^{99m}Tc and other isotopes (*5*). Among those radionuclides that are suitable for single-photon scintigraphy, ^{99m}Tc is considered the superior isotope for most imaging applications. The superiority of ^{99m}Tc as an imaging label for peptides also is predicted because its short (6-hr) physical half-life is an appropriate match with the pharmacokinetics of these molecules, in particular, their rapid rate of whole-body clearance (*5*).

Various and diverse strategies have been described for the labeling of peptides with ^{99m}Tc. For example, under certain circumstances, they may be radiolabeled directly without the prior attachment of an exogenous chelating group. Labeling in this manner usually accompanies the disruption of disulfide bridges within the molecule (*6*). These direct methods of labeling, therefore, will be successful only to the extent that this disruption is not harmful to biological properties, a consequence that may be viewed as unlikely in the case of small peptides, as compared to larger polypeptides or proteins. Another direct and more elegant method of labeling relies on the chelating ability of amino acids that are present in the native peptides or are added during synthesis (*7*). For example, the addition, through genetic engineering, of a Gly-Gly-Gly-Cys tetrapeptide on the C terminus of a peptide will provide a chelating site (*8*). This approach has the advantage of site direction of the chelator, but it cannot be applied to the labeling of preformed peptides.

Most investigators have preferred to prepare ^{99m}Tc-labeled peptides by the conventional use of bifunctional chelators, that is, chelating molecules with functional groups that permit their covalent conjugation to the peptide. One such bifunctional

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chelator is the N-hydroxysuccinimide (NHS) derivative of hydrazino nicotinamide ("hynic") (*9*). Although this chelator is unquestionably useful for labeling antibodies, the recent indication that ^{99m}Tc-hydrazino nicotinamide may be an undefined mixture of labeled species with affinities for nonspecific protein binding has tempered enthusiasm for this labeling method when it is applied to smaller molecules (*10,11*). Nevertheless, hynic has been used successfully to prepare radiolabeled chemotactic peptides for infection/inflammation imaging (*12*).

Other bifunctional chelators that are useful for the preparation of ^{99m}Tc-labeled antibodies also may be useful for radiolabeling small peptides (*2,3*). In particular, the N_3S amidothiol mercaptoacetyltriglycine (MAG3) has been shown to provide a stable label (*13*). This amidothiol is usually S-protected by a benzoyl group, which requires high temperatures or alkaline conditions for deprotection before radiolabeling. As a consequence, the chelate is normally preformed before conjugation in the case of temperature- or pH-sensitive proteins (*14*). In addition to a rather involved synthesis, the need to prelabel before conjugation has restricted the use of this MAG3. Recently, we developed a synthesis of a NHS ester of MAG3 (NHS-MAG3) chelator, in which the thiol is protected by an acetyl group (*15*). The NHS ester permits ready conjugation to primary amines, and the acetyl group protects during storage but apparently is sufficiently labile that deprotection is accomplished at ambient temperature and at neutral pH during labeling with ^{99m}Tc. We have established that the ^{99m}Tc MAG3 chelate is adequately stable for in vivo applications (*16,17*).

The objective of this investigation was to establish whether the S-acetyl NHS-MAG3 bifunctional chelator can be used to radiolabel a variety of small peptides with ^{99m}Tc. The four used in this research, each with 50–60 amino acid residues and molecular masses in the range 6–7 kDa, were: bovine pancreatic trypsin inhibitor (BPTI, or aprotinin), epidermal growth factor (EGF), human neutrophil elastase inhibitor EPI-HNE-2 (HNEI, a variant of BPTI) and plasmin inhibitor EPI-PLA-2 (PLAI) (*18*).

MATERIALS AND METHODS

Human recombinant EGF was purchased (Calbiochem, La Jolla, CA); the remaining three peptides were gifts from Dyax (Cambridge, MA). Cysteine, dimethylformamide, dicyclohexylcarbodiimide, 4,4′-dithiodipyridine, N-(2-hydroxyethyl)pipovazine-N′-(2-ethanesulfonicacid) (HEPES), stannous chloride dihydrate, triglycine, S-acetylthioglycolic acid NHS ester (Sigma Chemical Co., St. Louis, MO), disodium ethylenetriaminetetraacetic acid, mercaptoacetic acid and NHS (Aldrich Chemical Co., Milwaukee, WI) were purchased and used as received. The ^{99m}Tc-pertechnetate was obtained from a ^{99}Mo-^{99m}Tc radionuclide generator (DuPont, Billerica, MA). Dimethylformamide (DMF) was dried over molecular sieve.

Synthesis of N-Hydroxysuccinimide Mercaptoacetyltriglycine

The synthesis of NHS-MAG3 has been described previously (*15*). Briefly, S-acetyl MAG3 was prepared by adding S-acetylthioglycolic acid NHS ester in DMF, dropwise, to a solution of triglycine. After stirring, the solvents were evaporated, and the residue was washed with acetonitrile. The residue was purified over a silica gel column that was eluted with acetonitrile/methanol (80:20, vol/vol). Fractions containing MAG3 were combined. The solvents were evaporated under vacuum, and the residue was crystallized from isopropanol.

The synthesis of NHS-MAG3 from purified S-acetyl MAG3 was accomplished by mixing MAG3 and NHS in DMF and adding dicyclohexylcarbodiimide in DMF. The precipitated dicyclohexylurea was filtered and the solvent was evaporated. The residue was washed with isopropanol and further purified on a silica gel column with dry acetonitrile. The fractions containing NHS-MAG3 were combined, and the solvents were removed under vacuum. The residue was crystallized from isopropanol.

Peptide Conjugation and Radiolabeling

Each peptide was prepared at a concentration of 1.3–5 mg/ml in 0.1 *M* HEPES buffer (pH 8.0). A fresh solution of 10 mg/ml NHS-MAG3 in DMF, dried over molecular sieve, was added dropwise with mixing up to a MAG3-to-peptide molar ratio of 5:1–20:1. The solution was left undisturbed for 1 hr before purification over a 0.7 × 20 cm P-4 column (Bio-Rad, Melville, NY) eluted with 0.25 *M* ammonium acetate and 0.25 m*M* DTPA (pH 5.2). The peak fractions were collected and quantitated by ultraviolet (UV) absorption at 280 nm using extinction coefficients of 0.80 (BPTI), 3.42 (EGF), 0.89 (HNEI) and 1.15 (PLAI) for 0.1% solutions.

Each conjugated and purified peptide was typically present at a concentration of about 100 μg/ml in the 0.25 *M* ammonium acetate buffer. Aliquots from a 50 mg/ml solution of sodium tartrate in 0.5 *M* sodium bicarbonate, 0.25 *M* ammonium acetate and 0.175 *M* ammonium hydroxide (pH 9.2) were added until the final concentration of sodium tartrate reached 7 μg/μl. An aliquot from a fresh solution of stannous chloride in 10 m*M* HCl was then added so that the final stannous chloride dihydrate concentration was 1 μg per 10 μg of peptide. After the addition of ^{99m}Tc-pertechnetate in 3–5 μl, the solution was incubated at ambient temperature for 1 hr. The final pH was 7.6. The labeled peptides were purified over the P-4 column with 0.2 *M* phosphate-buffered saline (PBS) (pH 7.2) eluant. As controls, each native, unconjugated peptide was also subjected to the same labeling procedure.

After purification, radiochemical purity was determined by size-exclusion, high-performance liquid chromatography (HPLC) using a single 0.7 × 30 cm column of Superose-12 (Pharmacia, Piscataway, NJ) and 0.1 *M* sodium phosphate buffer (pH 7.0) as eluant. Radiochemical purity also was estimated by Sep-Pak C-18 (Waters, Milford, MA) column chromatography. A column was conditioned with 10 ml of absolute ethanol followed by 10 ml of 1 m*M* HCl (*19*). After the sample was loaded, the first elution, with 10 ml of 1 m*M* HCl, removed ^{99m}Tc-pertechnetate and ^{99m}Tc-tartrate. The second elution, with 10 ml ethanol/saline (1:1), removed the labeled peptide. Radiolabeled colloids remained on the column.

The specific activity of a labeled peptide may be an important consideration in cases in which the peptide is toxic (*12*). An estimate of maximum specific activity attainable with this MAG3 labeling method (at ambient temperatures and neutral pH) was obtained by adding 10 mCi ^{99m}Tc in 50 μl to 10 μg of peptide. Conditions were otherwise identical to those described above. After purification on the P-4 column, peptide recovery was determined by UV absorbance at 280 nm, and the specific activity was estimated from the radioactivity recovered.

Groups per Molecule

The average number of MAG3 groups per molecule was determined by using 4,4′-dithiodipyridine as an indicator of free thiols (*20*). All solutions were purged with N_2 before use. A solution of 2 m*M* 4,4′-dithiodipyridine in 0.1 *M* sodium phosphate (pH 7.2) was prepared. To construct a standard curve, a fresh 60-μ*M* solution of NHS-MAG3 in 0.25 *M* ammonium acetate and 0.25 m*M* DTPA (pH 5.2) buffer was prepared and serially diluted with the same buffer to concentrations of 50, 40, 30, 20, 10 and 5 μ*M*. A fresh 0.5-*M* solution of hydroxylamine HCl in 50 m*M* sodium phosphate and 25 m*M* ethylenetriaminetetraacetic acid (pH

7.5) was prepared, and 10 μl were added to 100 μl of each solution of MAG3 (i.e., 10% by volume) to deprotect the thiol. The solutions were kept at room temperature for at least 15 min, and then 100 μl of the indicator solution were added. The UV absorbance at 324 nm was read after 1 hr. In this way, a standard curve was constructed of UV absorbance compared to concentration of thiol groups. Both the native, unconjugated peptides and the conjugated peptides were then analyzed, and the absorbance was applied to the standard curve.

Stability Studies

Size-exclusion HPLC analysis with the Superose-12 column and 0.1 *M* sodium phosphate buffer (pH 7.0) eluant was used to estimate stability of each labeled peptide to incubation at 37°C in fresh human serum. A shift to higher molecular weight of the radioactivity profile signified serum protein binding while the presence of low molecular weight peaks signified breakdown to labeled catabolites.

Typically, about 1 μg of each radiolabeled peptide was added to 1 ml fresh human serum. The solutions were incubated at 37°C, and samples were removed at various times from 5 min to 24 hr.

Affinity Studies

As a further demonstration of specific radiolabeling in this investigation, each radiolabeled peptide was analyzed by HPLC before and after the addition of its binding protein. A shift in the radioactivity profile to a high molecular weight after the addition of the binding protein in solution (BPTI, HNEI and PLAI) or loss of radioactivity from the solution after the addition of immobilized binding protein (EGF) signifies that the radiolabel was bound to its peptide.

Bovine Pancreatic Trypsin Inhibitor. A tenfold molar excess of trypsin (Sigma) in a 1-mg/ml saline solution was added to radiolabeled BPTI in 0.2 *M* PBS. The solution was incubated at ambient temperature for 10–20 min before HPLC analysis.

Epidermal Growth Factor. A radiolabeled EGF sample was analyzed by HPLC before and after exposure to EGF receptor-positive A431 tumor cubes. The 1-mm^3 tumor cubes were cut from a A431 tumor that was freshly excised from a *nu/nu* Swiss mouse (*21*). To a solution containing 0.23 μg/ml labeled EGF was added a tumor cube, and the sample was incubated with agitation for 2 hr. The solution then was analyzed by HPLC, in which the same volume of supernatant was analyzed. Loss of radioactivity after incubation acted as an indication of specific binding.

Human Neutrophil; Elastase Inhibitor. A ten-fold molar excess of human neutrophil elastase (hNE; Calbiochem) was added to a preparation of radiolabeled HNEI in 10 m*M* sodium phosphate and 1 *M* sodium chloride (pH 7.5). After 1 hr, an HPLC radiochromatogram was obtained.

Plasmin Inhibitor. Labeled PLAI was added to buffer to a concentration of 6.6 μg/ml, and the mixture was analyzed by HPLC, before and at 3 hr after the addition of a sixfold molar excess of human plasmin (Calbiochem).

Cysteine Challenge

Each labeled peptide was tested for instability toward cysteine (*22*). The peptide concentration was adjusted to 2.2 μ*M* in 0.2 *M* PBS (pH 7.2). A freshly made solution of cysteine at 10 mg/ml in 0.1 *M* PBS (pH 7.0) was prepared and diluted in the first buffer to concentrations of 1, 0.1 and 0.01 mg/ml. Twelve microliters of each of these cysteine solutions were added to test tubes containing 90 μl of 2.2-μ*M* radiolabeled peptide. The molar ratios of cysteine to peptide, therefore, varied between 5:1 and 500:1. Each test tube was incubated for 1 hr at 37°C. After incubation, each solution was analyzed by Sep-Pak C-18 column chromatography. The column was prewashed with 10 ml ethanol followed by 10 ml of 0.1-m*M* HCl. After the sample was loaded, the column was rinsed with 10 ml of 1-m*M* HCl to elute labeled cysteine, tartrate and pertechnetate. The column was then eluted with 10 ml ethanol/saline (1:1, vol/vol) to recover the labeled peptide. Aliquots of both eluants were counted in a NaI(Tl) well counter.

Activity Measurements

Because of the unique nature of each peptide within this investigation, the methods used to measure biological activity after coupling and radiolabeling varied among the peptides.

Bovine Pancreatic Trypsin Inhibitor. Because of the very high affinity of BPTI for trypsin (~60 f*M*), the possibility exists that moderate decreases in affinity might not be detected in an assay based on trypsin inhibition. Accordingly, the activity of this peptide for plasmin inhibition was measured instead. An ambient temperature substrate hydrolysis assay was used to determine the apparent dissociation constant (K_i) for the inhibition of plasmin by BPTI (*23*). The BPTI was coupled with NHS-MAG3 at a MAG3-to-peptide molar ratio of 20:1.

The concentration of active sites in a sample of plasma was first established by titration using purified bovine BPTI (Miles, Inc., Kankakee, IL) under conditions ([plasmin] $\gg K_i$) in which BPTI functions as an effective irreversible inhibitor. The K_i was determined for MAG3-BPTI by measuring the residual plasmin activity that remained after incubation in the presence of the inhibitor. The percent residual plasmin activity is the ratio of the relative substrate hydrolytic activity that was obtained after incubation with BPTI to the relative activity obtained in the absence of BPTI, expressed as a percentage. The data were measured and fit as described previously (*23*).

Epidermal Growth Factor. Two EGF-positive cell lines, LS174T and A431, were obtained from American Type Culture Collection (Rockville, MD) and were grown in minimal essential culture medium (MEM) (Gibco, Grand Island, NY). The cells were removed from the culture flask by a brief incubation with an enzyme-free cell dissociation solution (Sigma) and resuspended in MEM containing 0.1% human serum albumin. Cells were counted using a trypan blue dye exclusion assay (*24*). To a constant cell number (10^5) was added between 1 and 1000 ng of labeled EGF per tube, in duplicate. After 30 min of incubation on ice, the cells were washed three times in 0.2 ml MEM containing 0.1% human serum albumin and pelleted by centrifugation for counting in a NaI(Tl) well counter. The study was repeated with cells preincubated with 2.5 μg of native EGF to saturate all EGF receptors. In each case, the percentage of added radioactivity that was bound to the cell pellet was recorded.

In addition, a competitive binding assay was performed in which 1.5–1000 ng of unlabeled but MAG3-coupled EGF and native EGF were added to a fixed number (10^5) of A431 cells or LS174T cells suspended in MEM. Radiolabeled EGF (1 ng) was added and, after 30 min of incubation, the cells were pelleted and counted as before.

Human Neutrophil Elastase Inhibitor. The affinity constant (K_i) of HNEI for hNE was measured for native HNEI inhibitor and for HNEI coupled at a MAG3-to-HNEI molar ratio of 1:1–50:1. The measurement of affinity was determined as described previously (*25*). Briefly, the ability of various concentrations of native or coupled HNEI to inhibit the cleavage of a fluorogenic substrate by hNE was measured, and the data were fitted by least squares, as described previously (*26*).

In addition, the kinetics of binding of labeled HNEI to immobilized hNE also was determined. Human neutrophil elastase (400 μg) was bound to 10 mg of 5-μm-diameter glyceryl microporous glass beads (CPG Corp., Lincoln Park, NJ), according to the manufacturer's instructions. The beads were incubated at 37°C in

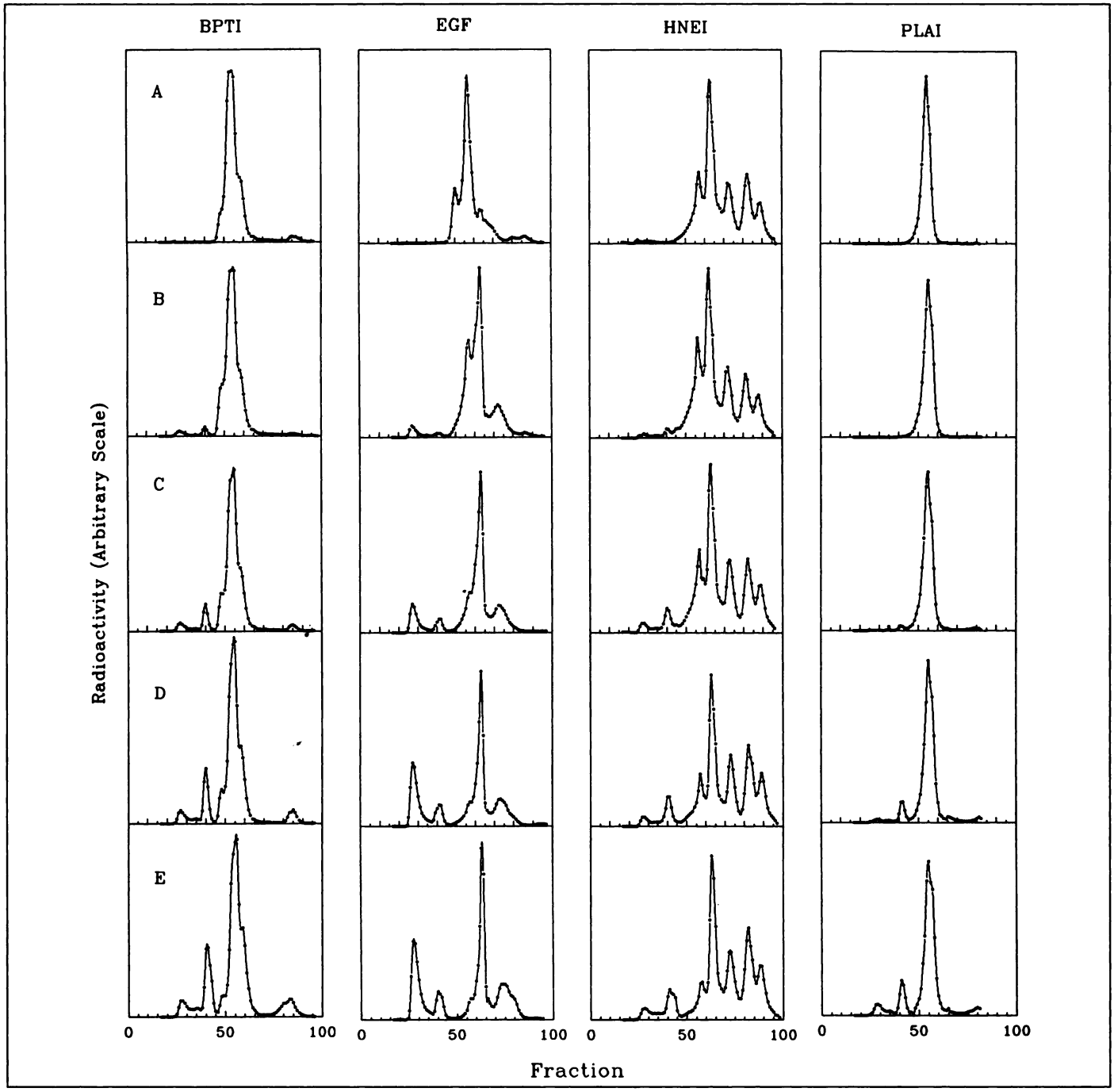

FIGURE 1. Size-exclusion HPLC radiochromatograms of ^{99m}Tc-labeled peptides (BPTI, EGF, HNEI and PLAI). Labeled peptides in (A) saline and (B) after 5 min, (C) 1–1.5 hr, (D) 4–5 hr and (E) 24 hr in 37°C serum.

fresh human serum, along with 0.13 μmol of labeled HNEI for 1–60 min. An aliquot was removed at each time point, and the beads were separated by filtration, rinsed and counted. An identical control study was performed using beads conjugated with glycine in place of hNE.

Plasmin Inhibitor. The effect of coupling with MAG3 on PLAI binding to plasmin and inhibitory activity was determined in a substrate hydrolysis assay. Briefly, plasmin, at a final active site concentration of 1.7 n*M*, and aliquots of various amounts of inhibitor (either native or MAG3-coupled PLAI) were mixed in 200 μl. After 90 min of incubation at ambient temperature, fluorogenic substrate [N-succinyl-Ala-Phe-Lys-7-amido-3-methyl-coumarin (Sigma)] was added to a final concentration of 100 μ*M*. After 10 min, the reaction was stopped with citrate buffer (pH 3.0). Fluorescence at 470 nm (with excitation at 380 nm) was measured. Data were measured and fit as before (*23*).

Biodistribution, Serum and Urine Analysis

Normal CD-1 male mice were injected through a tail vein with 0.1 ml of 0.2-*M* PBS containing 2–5 μg (100–200 μCi) labeled peptide. At 3 hr, animals were anesthetized with metofane and killed by cervical dislocation. Animals were dissected, tissues were rinsed in cold saline and counted, along with a standard of the injectate, in a NaI(Tl) well counter. Samples of serum and urine obtained from the animals at kill were analyzed by size-exclusion HPLC.

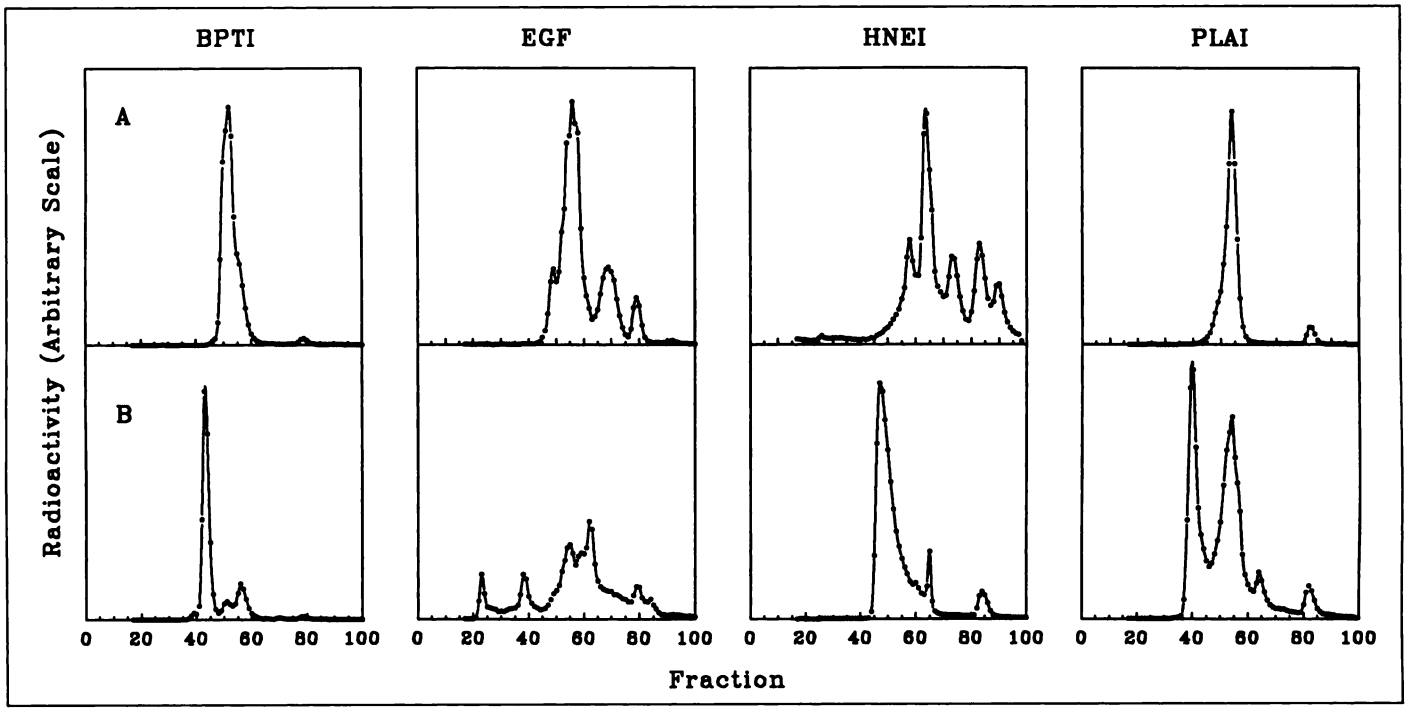

FIGURE 2. Size-exclusion HPLC radiochromatograms of ^{99m}Tc-labeled peptides (A) before and (B) after the addition of its binding protein, either in solution (BPTI, HNEI and PLAI) or bound to tumor cubes (EGF).

RESULTS

Peptide Conjugation and Radiolabeling

The conjugation of each peptide was performed under identical conditions and proceeded without difficulty in each case. Radiolabeling also was achieved without difficulty. Radiolabeling efficiency after 1 hr at ambient temperature for all four peptides averaged 54% (s.d. = 13%, n = 30). In contrast, the radiolabeling efficiency under identical conditions in the case of all four uncoupled control peptides was less than 5%. After purification, the radiochemical purity of each labeled peptide was always greater than 90%.

The above values for radiochemical labeling efficiency are based on the addition of about 50 μCi ^{99m}Tc per μg of peptide to about 100 μg of peptide, which provided a specific activity of about 20–30 μCi/μg. At the expense of labeling efficiency, the specific activity for all four peptides could be raised to about 3500 mCi/μmol through the addition of about 10 mCi ^{99m}Tc to 10 μg of peptide.

Groups per Molecule

The average number of MAG3 groups attached to each peptide was 1.4 (s.d. = 0.3, n = 14) and appeared to be independent of peptide.

Stability Studies

Figure 1 presents HPLC radiochromatograms of ^{99m}Tc-labeled BPTI, EGF, HNEI and PLAI. In each case, the uppermost row (A) is that of the radiolabeled peptides in saline, whereas all other radiochromatograms (B–E) were obtained by analysis of the labeled peptides after incubation in 37°C serum for 5 min to 24 hr. The recovery in each analysis varied between 80% and 100%. Labeled BPTI shows one major peak, with a slight shoulder in saline and in serum. This peak is still predominant, even after 24 hr in serum, although both higher and lower molecular weight peaks are apparent at this time. In the EGF case, one predominant peak and at least two smaller peaks are apparent in saline. After only 5 min in serum, one of these minor peaks becomes the predominant peak and persists as such throughout the 24 hr of incubation. At least five observable peaks are readily apparent in the case of HNEI, even in saline; however, unlike EGF, the peak intensities do not change appreciably with incubation in serum over 24 hr. Finally, the radiochromatograms of labeled PLAI show one peak through 75 min, which predominates with only a slight shoulder, even through 24 hr.

Affinity Measurements

Figure 2 presents HPLC radiochromatograms of labeled peptides (A) before and (B) after the addition of the respective binding peptide, either in buffer (BPTI, HNEI and PLAI) or on tumor cubes (EGF). In the former case, binding will be evident

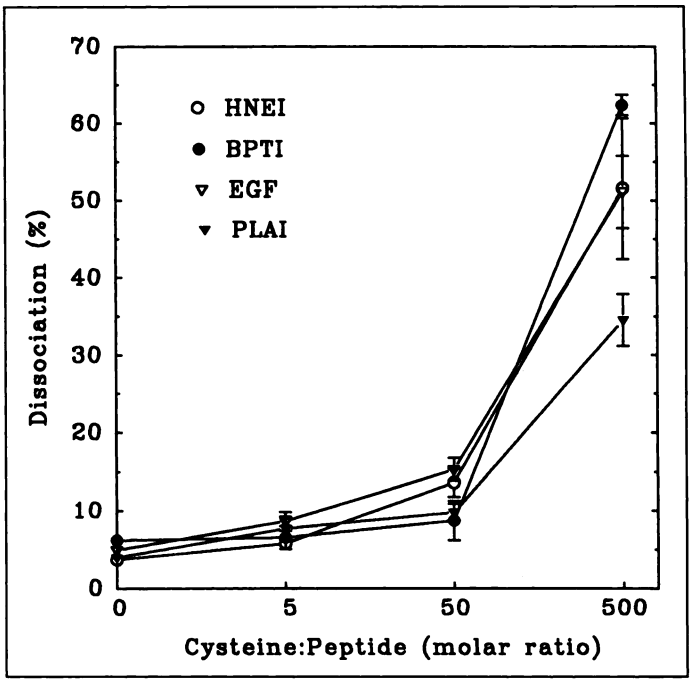

FIGURE 3. Cysteine challenge studies of ^{99m}Tc-labeled peptides. Results presented as percentage dissociation compared to cysteine molar ratio. Error bars show range of repeat measurements (n = 3).

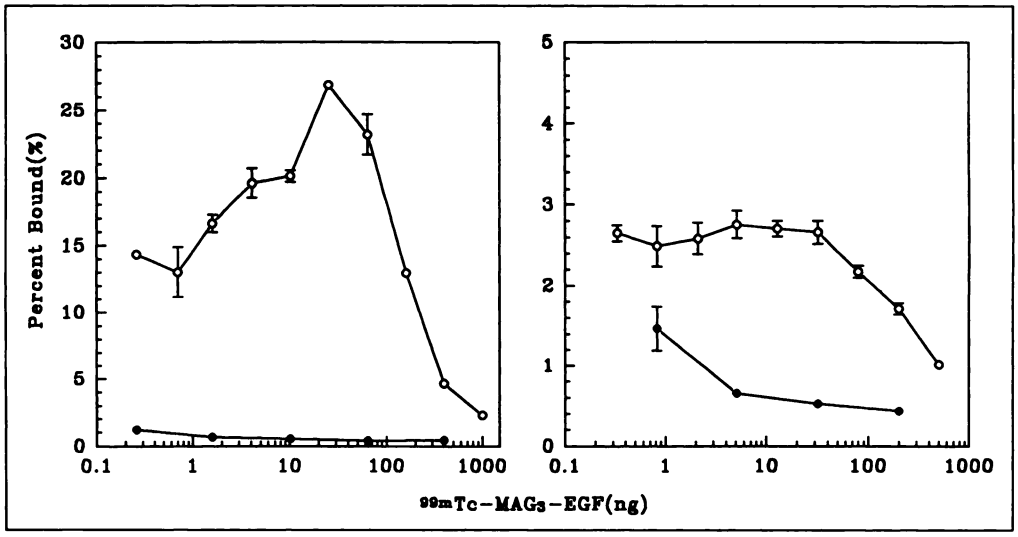

FIGURE 4. Percentage of ^{99m}Tc-labeled EGF bound to A431 cells (left) and LS174T cells (right) after incubation with increasing concentrations of labeled EGF (○). Results are also presented for EGF-saturated cells (●). Error bars show range of repeat measurements (n = 2).

by a shift in the radiochromatographic profile to higher molecular weight (i.e., earlier retention times) and, in the latter case, by a loss of radioactivity from the sample.

In the case of most peptides studied, a majority of the radiolabel has been shown to be attached to active peptide. In the BPTI case, the addition of trypsin has resulted in a large shift in the radioactivity profile to earlier fractions. The size-exclusion HPLC radiochromatograms of labeled EGF, before and after exposure to EGF-positive tumor cubes, show a large loss of radioactivity from solution (the same volume of supernatant was injected in each EGF analysis and recoveries were identical). Each of the major peaks has disappeared in a demonstration that each was a form of labeled EGF. Figure 2 also shows the effect on radiolabeled HNEI after the addition of a tenfold molar excess of hNE. The complete shift of radioactivity to earlier retention times demonstrates that all identifiable peaks in the HNEI radiochromatogram are due to labeled and viable HNEI.

The last column in Figure 2 presents radiochromatograms showing labeled PLAI after 3 hr in 0.25 *M* ammonium acetate (pH 5.2) (A) and after the addition of a sixfold molar excess of plasmin (B). Recoveries were 95%. Approximately half the radioactivity shifted to a higher molecular weight after the addition of plasmin. Accordingly, in the case of PLAI, only about half the radioactivity was on active peptide.

Cysteine Challenge

Figure 3 presents the results of cysteine challenge for all four labeled peptides. The results are presented as the percentage of the radiolabel transferring to cysteine at cysteine-to-peptide molar ratios in the range of 0:1–500:1. Error bars show the range of repeat measurements (n = 3) for a single preparation of each labeled peptide.

The instability toward cysteine transchelation is similar for all four peptides, reaching, in this assay, about 50%–60% dissociation after 1 hr in 37°C cysteine solution at a 500:1 molar ratio.

Activity Measurements

Bovine Pancreatic Trypsin Inhibitor. A plot of residual plasmin activity compared to MAG3-BPTI showed no decrease with increasing concentration (data not presented) and was used to calculate a K_i of 31 p*M* for the conjugated BPTI. This is in contrast to 1.5 n*M* for inhibition of plasmin by native BPTI (*18*) and signifies that, regarding plasmin inhibition, the peptide was almost completely denatured by the conjugation.

Epidermal Growth Factor. Figure 4 shows the percentage of radiolabel bound to A431 cells (left) and LS174T cells (right) after incubation with increasing concentrations of radiolabeled EGF (open circles). In each case, results also are presented for cells presaturated with EGF (filled circles). Each data point

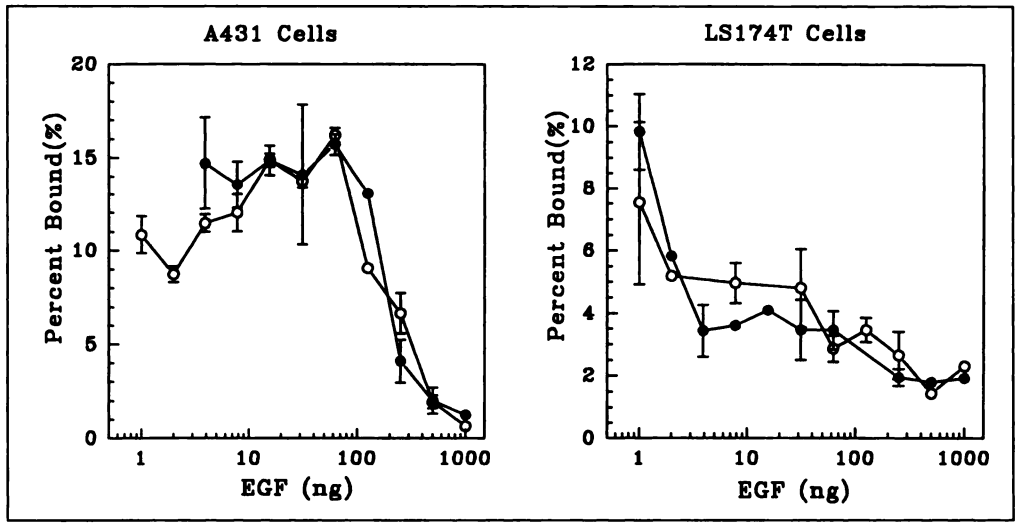

FIGURE 5. The percentage of radiolabeled EGF bound to a constant number of A431 cells (left) and LS174T cells (right) with increasing unlabeled native (○) and MAG3-coupled EGF (●). Each data point represents the mean of repeat measurements. Error bars show the range of values obtained.

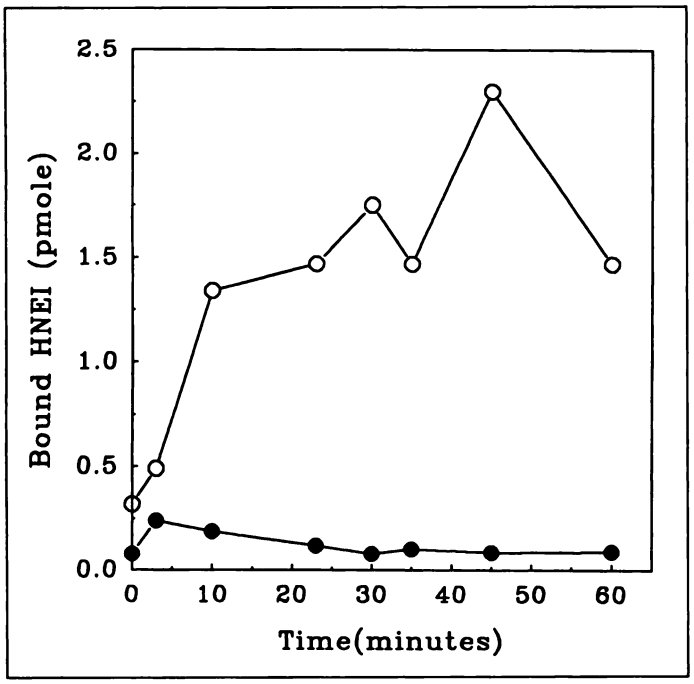

FIGURE 6. The kinetics of binding of labeled HNEI to beads conjugated with hNE (○) and to control beads (●).

represents the mean of repeat determinations on two preparations, with the error bars indicating the range of values. A maximum of about 25%–30% of the labeled EGF was bound to A431 cells before this percentage decreased with increasing EGF due to receptor saturation. In contrast, cells presaturated with EGF showed essentially no binding at all EGF concentrations. Results with LS174T cells were similar, although the percentage that was specifically bound was lower.

Figure 5 presents the results of a competitive binding assay that measures the extent to which labeled EGF will bind to a fixed number of A431 cells (left) and LS174T cells (right) as the amount of unlabeled EGF is increased. The measurement was performed for MAG3-coupled EGF (filled circles) and native EGF (open circles). Each data point represents the mean of repeat measurements, whereas error bars show the range of values obtained. Throughout the range of the measurement, no important differences were apparent between coupled and native EGF in an ability to compete with native EGF for the EGF receptors on A431 cells and on LS174T cells.

Human Neutrophil Elastase Inhibitor. The equilibrium constant K_i for native HNEI was found to be 1.2 p*M*, whereas the k_{on} and k_{off} constants were determined to be 4.7 × (10^6/N-sec) and 5.8 × (10^{-6}/sec), respectively. These values for HNEI coupled at a MAG3-to-peptide molar ratio of 1:1 were 0.7, 6.0 and 4.0, respectively. When coupled at at 50:1 molar ratio, these values were 1.1, 5.2 and 5.7, respectively. These differences are within the limits of the measurement and are not significant.

Figure 6 shows the results of measuring the kinetics of binding of labeled HNEI to hNE immobilized beads (open circles) and to control beads without hNE (filled circles). At 37°C, in serum and under the conditions of this measurement, binding appeared to be completed in about 30 min and is specific to hNE.

Plasmin Inhibitor. The equilibrium constants for plasmin binding were found to be 89 p*M* for native PLAI and 95 p*M* for PLAI conjugated with MAG3 at a MAG3-to-PLAI molar ratio of 20:1. This difference is considered to be within the error of the measurement.

Biodistribution, Serum and Urine Analysis

Table 1 lists the biodistribution results of all four labeled peptides, 3 hr after intravenous administration to normal mice. Important differences are readily apparent: liver levels were much higher for BPTI and EGF, as compared to HNEI and PLAI. Kidney levels of BPTI were much higher than those for the remaining peptides, and stomach and small intestine levels were highest for EGF.

Figure 7 presents size-exclusion HPLC radiochromatograms of each labeled peptide in saline (A) and in serum (B) and urine (C), both of which were obtained 30 min after intravenous administration of the radiolabeled peptide to normal mice. As is evident from Figure 7 and in agreement with Figure 1, each peptide showed some tendency to bind to serum proteins. Accelerated clearance of the free peptide relative to the serum-bound peptide in vivo would explain the higher concentration of radiolabel on serum proteins in vivo (Fig. 7) compared to that in vitro (Fig. 1).

DISCUSSION

This investigation has demonstrated that peptides may be successfully radiolabeled at ambient temperature and neutral pH with ^{99m}Tc using the bifunctional chelator NHS-S-acetyl-MAG3. That the chelating groups were labeled was shown by radiolabeling efficiencies, which averaged about 50% for all four conjugated peptides, as compared to an average of less than 5% for the unconjugated native peptide. Under optimal conditions, specific activities as high as 3500 mCi/μmol were achieved for each labeled peptide. Specific activities in this range should be acceptable for imaging applications (*12*). Furthermore, higher labeling efficiencies and specific activities

TABLE 1
Biodistribution of Technetium-99m-Labeled Peptides at 3 hr after Intravenous Administration to Normal Mice*

Tissue	HNEI	PLAI	BPTI	EGF
Liver	0.39 (0.06)	0.07 (0.04)	3.6 (0.4)	2.7 (0.2)
Heart	0.07 (0.01)	0.01 (0.00)	0.16 (0.02)	0.12 (0.01)
Kidney	14.0 (3.0)	3.5 (0.7)	56.0 (10.1)	4.6 (0.7)
Lung	0.39 (0.18)	0.04 (0.01)	0.48 (0.09)	0.22 (0.03)
Stomach	0.53 (0.23)	0.37 (0.25)	0.10 (0.07)	3.5 (1.2)
Spleen	0.11 (0.02)	0.02 (0.01)	0.43 (0.12)	0.20 (0.03)
Small intestine	0.18 (0.07)	0.31 (0.27)	0.87 (1.2)	2.1 (2.0)
Muscle	0.06 (0.02)	0.01 (0.00)	0.11 (0.04)	0.07 (0.02)
Blood	0.24 (0.04)	0.05 (0.01)	0.27 (0.05)	0.32 (0.03)
n	6	5	5	5

*Results are presented as percentage injected dose per g, with 1 s.d. in parentheses.

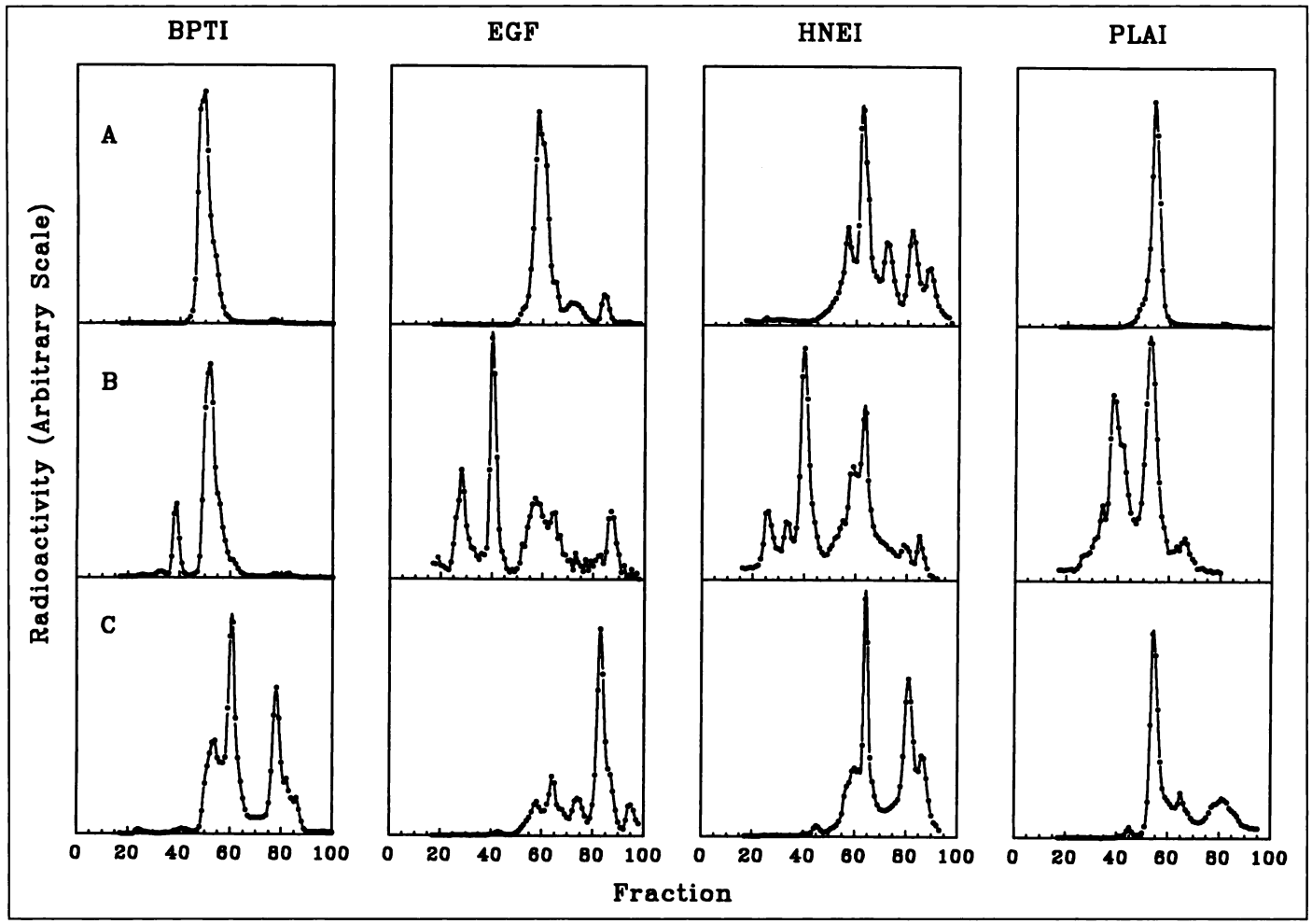

FIGURE 7. Size-exclusion HPLC radiochromatograms of each labeled peptide in (A) saline and in (B) serum and (C) urine, obtained 30 min after intravenous administration of the radiolabeled peptide to normal mice.

may be achievable in the case of peptides that are capable of withstanding higher temperatures or alkaline pH during labeling (*27*).

Using a variety of activity measurements, no evidence of denaturation of HNEI and EGF was observed. Specifically, coupled EGF was found to compete for EGF receptor-positive cells equally as well as did native EGF (Fig. 5). This labeled peptide also showed specific binding to these cells (Fig. 4). Labeled HNEI, despite displaying multiple peaks on HPLC analysis, shifted quantitatively in the presence of hNE (Fig. 2) and showed fairly rapid binding kinetics to hNE-bound beads (Fig. 6).

Essentially quantitative binding of MAG3-BPTI to excess trypsin was observed in this investigation (Fig. 2). By this measurement, the MAG3-BPTI was not denatured. However, plasmin also is known to be inhibited by BPTI and, because of much lower binding affinity, plasmin may be used in a more sensitive assay of affinity for protease inhibition. By this assay, it was possible to show an essential absence of plasmin inhibition by MAG3-BPTI. This peptide contains four lysine residues in positions 15, 26, 41 and 46 (*28*). The lysine in position 15 is directly in the middle of the peptide–inhibitor interface, such that altering this lysine is likely to abolish inhibitory activity. Alteration of the remaining three lysines should be less critical. Because BPTI in this research was conjugated with a twentyfold molar excess of NHS-MAG3, it is likely that all lysines in most BPTI molecules were modified, with the result that complete loss of plasmin inhibitory activity should be expected. It is interesting that this residue has been replaced in HNEI with an asparagine, such that HNEI cannot be modified at this critical position, and this may explain why no alteration in hNE inhibitory activity was observed in the case of MAG3-HNEI.

In the case of PLAI, conjugation with MAG3 at a MAG3-to-peptide ratio of 20:1 produced a functionally heterogeneous population. About half the coupled molecules retained full affinity for plasmin, as demonstrated by K_i measurements. The remainder of the MAG3-conjugated molecules apparently lost all (or almost all) affinity for plasmin (Fig. 2). Similar to BPTI, PLAI has a lysine at a position on the edge of the PLAI–plasmin interface, where MAG3 conjugation could possibly interfere with receptor binding.

One feature of this investigation was the observance of multiple peaks in the HPLC radiochromatograms, in particular, HNEI but also, to a lesser extent, most of the other peptides as well. Only PLAI presented essentially as a single peak on HPLC analysis. Nevertheless, it was possible to show conclusively that each peak in the HNEI radiochromatogram was due to a labeled HNEI by demonstrating a shift in the radioactivity distribution with the addition of hNE (Fig. 2). To investigate the nature of these peaks, one major peak eluting off the size-exclusion HPLC column in the analysis of radiolabeled HNEI was collected and reinjected. The new radiochromatogram showed features similar to that of the original chromatogram (data not presented). Thus, it is likely that the multiple peaks in

the HNEI case were due less to multiple conjugations than to charging or lipophilic effects on the HPLC column.

The serum stability of the label and the absence of significant serum protein binding was evident from the serum incubation studies of the labeled peptides (Fig. 1). In most cases, the radiochromatograms changed little from saline to 24 hr in 37°C serum. The sole exception was radiolabeled EGF, which showed an immediate shift of the radiolabel to lower molecular weight in serum. However, each peak in the EGF radiochromatogram was shown to be due to labeled EGF (instead of, for example, catabolites) because each was removed almost quantitatively from serum by exposure to EGF receptor-positive tumor cubes (Fig. 2). Thus, for each labeled peptide, little evidence of instability in serum leading to high molecular weight aggregation, serum protein binding or low molecular weight catabolites was evident.

The biodistribution of the radiolabeled peptides showed large variations among the peptides in some tissues (Table 1). In general, tissue levels were lower for PLAI in comparison to the other peptides. Labeled BPTI and EGF showed levels 10–50 times higher than those of HNEI and PLAI in liver, whereas in kidneys, labeled BPTI provided organ levels that were more than 10 times higher than those of PLAI and EGF. These differences would be unexpected in the case of an unstable radiolabel.

Finally, the labeled peptides each displayed a stability to cysteine challenge that was broadly independent of the peptide and typical of that observed by us for other carriers radiolabeled with ^{99m}Tc using NHS-S-acetyl-MAG3 (*16*).

CONCLUSION

It appears that NHS-S-acetyl-MAG3 is a convenient bifunctional chelator for the conjugation of peptides and subsequent radiolabeling with ^{99m}Tc. The bifunctional chelator is stable in long-term dry storage at ambient temperatures (*15*). By derivitizing with an NHS ester, familiar NHS coupling strategies may be used to attach MAG3 to peptides and to other amines. Finally, presumably because of the use of an acetyl group for sulfur protection, the radiolabeling may now be accomplished at ambient temperatures and at neutral pH (*29*). As demonstrated in this investigation, four peptides have been successfully radiolabeled using this strategy, and each has shown stability in serum and to cysteine challenge, which should be suitable for radiopharmaceutical applications.

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