

# Identification of the Soluble *in Vivo* Metabolites of Indium-111-Diethylenetriaminepentaacetic Acid-D-Phe<sup>1</sup>-Octreotide

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Indium-111-diethylenetriaminepentaacetic Acid-D-phenylalanine<sup>1</sup>-octreotide (<sup>111</sup>In-DTPA-octreotide) is a cyclic eight amino acid somatostatin analogue which is approved for gamma scintigraphy of neuroendocrine tumors. To address the factors that contribute to liver and kidney retention of this radiopharmaceutical, its metabolism was evaluated in normal and tumor-bearing rats. The soluble fractions from nontarget (liver and kidney) and target (tumor, pancreas, adrenals) organ homogenates were analyzed out to 21 h postinjection, and urine was analyzed out to 12 h postinjection. The blood was analyzed at shorter time intervals due to the rapid clearance of <sup>111</sup>In-DTPA-octreotide. Radio-TLC and HPLC were used to analyze organ homogenates, blood, and urine. By TLC, intact <sup>111</sup>In-DTPA-octreotide was resolved from the soluble metabolites, and a similar apparent rate of metabolism was observed in the liver, kidney, tumor, and pancreas with ~30% intact <sup>111</sup>In-DTPA-octreotide at 4 h postinjection. In the adrenals, metabolism occurred more slowly with ~60% intact <sup>111</sup>In-DTPA-octreotide at 4 h postinjection. At 4 h postinjection, the activity excreted in the urine consisted of 85% intact <sup>111</sup>In-DTPA-octreotide. HPLC provided resolution of the individual extractable metabolites. In an attempt to identify these metabolites, two DTPA-amino acid sequences were synthesized: DTPA-D-Phe-Cys and DTPA-D-Phe. Under the conditions used for metabolite analysis, <sup>111</sup>In-DTPA-D-Phe-Cys-OH eluted at 14.6 min and <sup>111</sup>In-DTPA-D-Phe-OH eluted at 7.0 min. Each of these standard sequences was combined with the soluble portion of the organ homogenate and was shown by HPLC to coelute with the metabolites. These data suggest that <sup>111</sup>In-DTPA-octreotide was initially degraded to <sup>111</sup>In-DTPA-D-Phe-Cys-OH and <sup>111</sup>In-DTPA-D-Phe-OH. The <sup>111</sup>In-DTPA-D-Phe-Cys-OH was further degraded to <sup>111</sup>In-DTPA-D-Phe-OH, which appeared to be the final metabolite that was extracted from the organs. From these results, it can be concluded that at longer time points (>2 h postinjection) a significant amount of <sup>111</sup>In was retained in nontarget organs as <sup>111</sup>In-DTPA-D-Phe-OH and <sup>111</sup>In-DTPA-D-Phe-Cys-OH and not as intact <sup>111</sup>In-DTPA-octreotide.

## INTRODUCTION

Somatostatin is a naturally occurring peptide hormone consisting of 14 or 28 amino acids. It is present in the hypothalamus, the cerebral cortex, the brain stem, the gastrointestinal tract, and the pancreas and exerts an inhibitory effect on several cell functions such as secretion of peptide hormones and growth factors (1–4). Neuroendocrine tumors have been found to have an increased number of somatostatin receptors. Other common types of tumors also contain somatostatin receptors, such as those of the CNS, breast, and lung (5–10). The clinical value of somatostatin is limited due to its very short half-life *in vivo* ( $t_{1/2} < 3$  min) (11). Octreotide, an eight amino acid peptide, is an analogue of somatostatin that is highly resistant to degradation by enzyme attack and more effective than the native hormone in the suppression of growth hormone secretion (12).

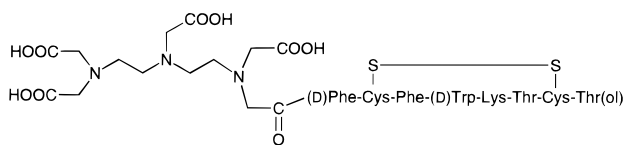
Somatostatin analogues, including octreotide have been labeled with a variety of radiometals such as <sup>111</sup>In (13, 14), <sup>64</sup>Cu (15), <sup>67/68</sup>Ga (16, 17), <sup>90</sup>Y (18), and <sup>188</sup>Re (19) for targeted radiotherapy or for imaging of somatostatin receptor positive tumors by SPECT or PET. Radiometals are linked to octreotide via a bifunctional chelate. The bifunctional chelate contains a functional group through which it is conjugated to octreotide. The chelate is chosen such that it binds the metal strongly to prevent release of the metal *in vivo*. The biodistribution of a variety of octreotide conjugates has been investigated in tumor-bearing rodents, and although receptor-mediated tumor uptake was observed, many of these agents exhibited high nontarget uptake in the kidney and liver. To alleviate nontarget accumulation, research efforts have focused on altering the stability of the metal–chelate complex or the chemistry of the chelate–octreotide bond.

Understanding the mechanism of uptake, degradation and retention of octreotide conjugates is key to designing optimal somatostatin analogues. Since <sup>111</sup>In-DTPA-octreotide<sup>1</sup> (Figure 1) is clinically used for the scintigraphic imaging of neuroendocrine tumors (20–23) and, more recently, has been studied for its therapeutic efficacy (11,

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**Figure 1.** Structure of <sup>111</sup>In-DTPA-octreotide.

24), we studied its metabolism *in vivo* in normal and tumor-bearing rats.

There have been few studies on the metabolism of radiolabeled octreotide analogues, although many reports have been published on the *in vivo* fate of various <sup>111</sup>In-labeled polypeptides and glycoproteins (25–30). It has been demonstrated that radiolabeled antibodies bound to cell surface antigens are internalized and delivered to the lysosome for degradation (31–34). Recent studies with liver-targeted <sup>111</sup>In-DTPA-glycoproteins have shown delivery of these molecules to lysosomes with subsequent degradation of the polypeptide backbone to a low molecular weight species. Chromatographic methods were used to identify <sup>111</sup>In-DTPA-lysine as this low molecular weight metabolite (25, 26, 35). <sup>111</sup>In-DTPA-lysine was also identified as the metabolic product of <sup>111</sup>In-DTPA-MAb conjugates in the liver and kidney (30). These results indicate that the DTPA–amino acid linkage (amide or thiourea bond) is stable *in vivo* and <sup>111</sup>In is not significantly dissociated from DTPA. It was therefore proposed that retention of radioactivity in the nontarget organs was due to the inability of In-DTPA-lysine to be released from the cell using the carrier-mediated transport systems designed for the intact polypeptide/antibody.

The internalization of <sup>111</sup>In-DTPA-octreotide has been evaluated by several research groups (11, 36, 37). Bree-man et al. injected <sup>111</sup>In-DTPA-octreotide into rats and at various times postinjection challenged receptor uptake with cold octreotide (36). He concluded that <sup>111</sup>In-DTPA-octreotide was rapidly internalized (<20 min) in somatostatin receptor positive tissues and normal organs. Recently, Duncan et al. confirmed that <sup>111</sup>In-DTPA-octreotide was rapidly internalized and, using subcellular fractionation techniques, identified lysosomes as the site of delivery in pancreatic, tumor, kidney, and liver cells (37). Andersson et al. studied the internalization and release of <sup>111</sup>In-DTPA-octreotide in human glucagonoma and carcinoid cells (11). Electron microscope autoradiography was used to determine the subcellular distribution of <sup>111</sup>In in the midgut carcinoid and activity was reported to be primarily in the cytoplasm, with about 30% found in the nucleus.

In the present study, the metabolism of <sup>111</sup>In-DTPA-octreotide was evaluated *in vivo* in target (somatostatin receptor containing) and nontarget organs of normal and tumor-bearing rats. The aims of this investigation were (1) to analyze and compare metabolism patterns in target and nontarget tissues; (2) to identify the retained radiolabeled species; and (3) to gain an understanding of the factors that contribute to nontarget retention of octreotide radiopharmaceuticals.

## MATERIALS AND METHODS

**Materials.** DTPA-octreotide and <sup>111</sup>InCl<sub>3</sub> (4.2 × 10<sup>5</sup> Ci/g) were provided by Mallinckrodt Inc. (St. Louis, MO). DTPA was purchased from Sigma Chemical Co. (St. Louis, MO). Trifluoroacetic acid (TFA) was purchased

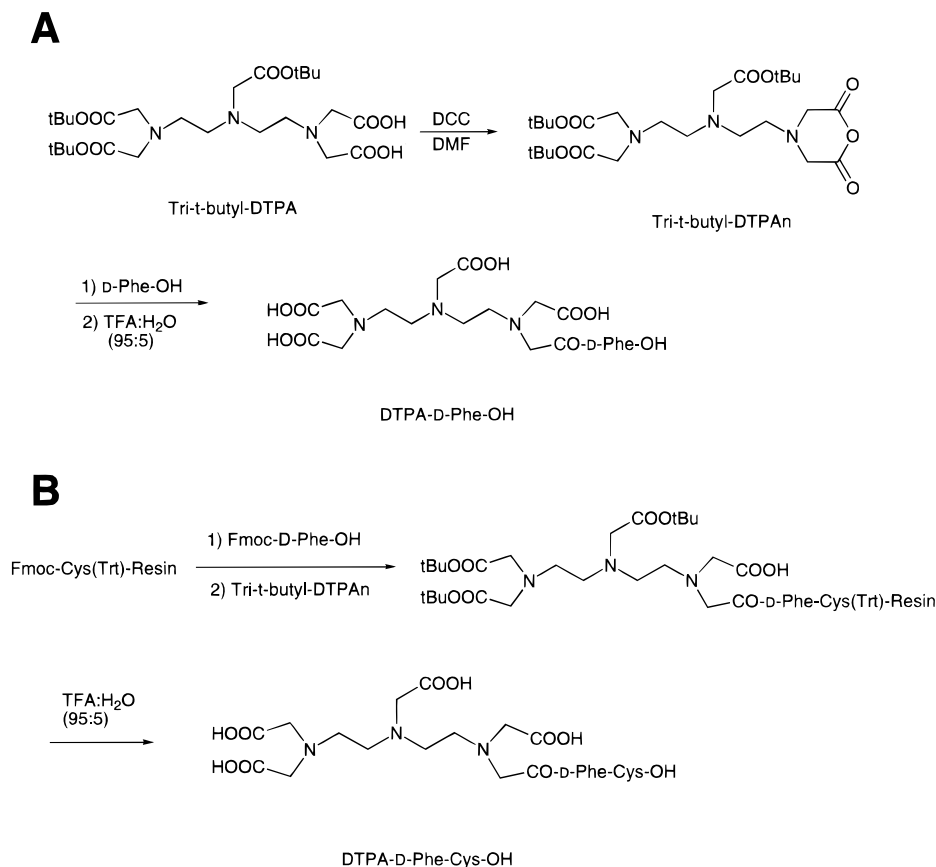
from J. T. Baker (Chicago, IL). C-18 Sep-Pak cartridges were from Waters/Millipore (Burlington, MA). Biospin-6 chromatography columns were from Bio-Rad Laboratories (Hercules, CA). Organs were prepared using a Tekmar tissue homogenizer (Cincinnati, OH), a Branson Sonifier 185 cell disrupter, and a Sorvall RC2-B centrifuge. Ultrafree-MC 0.22 μm filter units were from Millipore (Bedford, MA). Centricon-10 and -30 ultrafiltration units were from Amicon (Beverly, MA). Reversed-phase HPLC was performed on a Waters 600 system with a Waters 991 photodiode array detector (200–300 nm) and an Ortec (EG&G Instruments) radioactive detector. Reversed-phase columns used were either Vydac 218TP54 (C18, 4.6 × 250 mm, analytical) or Vydac 218TP1010 (C18, 10 × 250 mm, semi-prep). Unless otherwise indicated, a gradient elution was employed with 5% A to 70% A in 30 min (solvent A = 95% ACN:5% H<sub>2</sub>O and solvent B = 95% H<sub>2</sub>O:5% ACN, both solvents contained 0.1% TFA). Millenium software (Waters Corporation) was used to quantify chromatograms by integration. Reversed-phase silica gel (KC18F, 60 Å, 200 μm) TLC plates were from Whatman and the radioactive compounds were analyzed on a Bioscan System 200 imaging scanner after development in 70% MeOH:30% (5% NH<sub>4</sub>OAc). Radioactivity was quantified using a Beckman Gamma 8000 automated well-type gamma counter. Adult, female, Sprague–Dawley rats were purchased from Sasco (Omaha, NE), and female Lewis rats were purchased from Charles River Laboratories (Boston, MA). The rat pancreatic tumor CA20948 was obtained from the Tumor Bank at Biomeasure, Inc. (Hopkinton, MA). All animal experiments were performed in compliance with guidelines specified by the Washington University Animal Studies Committee.

**Synthesis of DTPA-D-Phe-OH and DTPA-D-Phe-Cys-OH.** Preparation of tri-*tert*-butyl-DTPA and a general method for the incorporation of DTPA by solid phase were previously described (38).

**DTPA-D-Phe-OH (Figure 2A).** To a solution of tri-*tert*-butyl-DTPA (42 mg, 75 μmol) in 1 mL of anhydrous dimethylformamide 15.5 mg of dicyclohexylcarbodiimide in 500 mL was added and shaken for 30 min. A solution of 8 mg (50 μmol) of D-phenylalanine in 1 mL of dimethylformamide containing 10 mg (12 mL, 100 μmol) of triethylamine was added and the solution was shaken for 3–4 h at room temperature. Dimethylformamide was removed *in vacuo*, and the residue was treated with 95% trifluoroacetic acid:5% water to remove the *tert*-butyl groups. The solvents were removed *in vacuo* and DTPA-D-Phe-OH was isolated by preparative reversed-phase liquid chromatography [mobile phase, solvent A (water: 0.1% TFA) and solvent B (90% H<sub>2</sub>O:10% acetonitrile:0.1% TFA); gradient, 95% A:5% B to 30% A:70% B in 40 min, linear gradient, detection mode UV at wavelength 214 nm] followed by lyophilization. DTPA-D-Phe-OH eluted at 6.1 min. MS, *m/z* 541.2 (M + 1).

**DTPA-D-Phe-Cys-OH (Figure 2B).** Solid-phase peptide synthesis (SPPS) was carried out using an Applied Biosystems Model 432 A “Synergy” Peptide synthesizer using Fmoc (9-fluorenylmethoxycarbonyl) strategy (39). The instrument protocol required 25 μmol of preloaded Fmoc amino acid to the resin and 75 μmol of subsequent Fmoc-protected amino acids. Activation was accomplished by a combination of hydroxybenzotriazole (HOBt) and 2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). Tri-*tert*-butyl DTPA (75 μmol) was placed at the appropriate location in the synthesizer and activated similar to the other amino acids for coupling. Fmoc-Cys(S-Trt)-Wang resin (50 mg,

<sup>1</sup> Abbreviations: DTPA, diethylenetriaminepentaacetic acid; SPECT, single-photon emission computerized tomography; PET, positron emission tomography; CNS, central nervous system.



**Figure 2.** Synthesis of metabolite standards. (A) Synthesis of Tri-*tert*-butyl-DTPAn and DTPA-D-Phe-OH; (B) synthesis of DTPA-D-Phe-Cys-OH.

0.5 mmol/g) was used. After successive coupling of Fmoc-D-Phe and tri-*tert*-butyl-DTPA, the peptide was cleaved and deprotected from the resin with trifluoroacetic acid: thianisole:phenol:water (85:5:5:5) for 8–10 h. The peptide was precipitated by the addition of 10 mL of *tert*-butylmethyl ether. Peptide-resin mixture was washed with 4 × 10 mL of *tert*-butylmethyl ether. Acetonitrile:water (2:3) was added to dissolve the peptide and the solution was filtered to remove the resin. The solution containing the crude peptide was lyophilized prior to purification by liquid chromatography. Final purification was accomplished by C-18 reversed phase chromatography [mobile phase, solvent A (water:0.1% TFA) and solvent B (90% H<sub>2</sub>O:10% acetonitrile:0.1% TFA), gradient, 90% A:10%B to 30% A:70% B in 40 min, linear gradient, detection mode UV at wavelength 214 nm] followed by lyophilization. DTPA-D-Phe-Cys-OH eluted at 9.3 min. MS, *m/z* 644.2 (M + 1).

**Radiolabeling.** <sup>111</sup>In-DTPA-octreotide was prepared as previously described by Anderson et al. and was analyzed by radio-TLC and HPLC (15). DTPA, DTPA-D-Phe, and DTPA-D-Phe-Cys (1–3 μg) were dissolved in NH<sub>4</sub>OAc, pH = 5.5, 0.1 M, and incubated with <sup>111</sup>Indium-acetate (1–3 mCi) at room temperature for 1 h. The compounds were analyzed by radio-TLC and HPLC without purification. Transferrin was also labeled by its incubation with <sup>111</sup>Indium-acetate. It was purified from uncomplexed <sup>111</sup>In by passage through a Biospin-6 column and was analyzed by radio-TLC.

**In Vivo Metabolism of <sup>111</sup>In-DTPA-Octreotide.** Mature, female, Sprague–Dawley rats and female Lewis tumor-bearing rats (*n* = 2–4) were injected with 200–2000 μCi of <sup>111</sup>In-DTPA-octreotide (specific activity, 1000–4000 μCi/μg) via the tail vein. Prior to injection, the dose

was diluted with 0.1% BSA in buffer or saline. At 1, 2, 4, 6, 8, 14, and 21 h postinjection the urine was collected and the liver, kidney, adrenals, pancreas, tumor, and blood removed. In the experiments, only a section of the liver was used (~1 g). All organs were rinsed with water and blotted dry. Samples were kept in an ice bath at all times.

To the organs and blood, 2.0 mL of 20% HEPES (1.0 M, pH = 7) in EtOH was added and the samples were homogenized (1–3 min), sonicated (30 s), and centrifuged (23000*g* for 10 min, 10 °C) to precipitate protein. This extraction was repeated with 1 mL of 20% HEPES in EtOH and the supernatants combined. Both supernatant and pellet were counted to determine extraction efficiency. Supernatants were passed through a 0.22 μm Ultra-MC spin filter and analyzed by HPLC and/or TLC. Organ blanks, where the <sup>111</sup>In-DTPA-octreotide injectate was added directly to tissues prior to workup, were performed as controls.

In separate experiments, four female Sprague–Dawley rats were injected with 2 mCi <sup>111</sup>In-DTPA-octreotide. At 4 h postinjection, the rats were sacrificed and their livers and kidneys excised. A portion of the livers and one kidney were homogenized and extracted twice with 20% HEPES in EtOH as described above. The pellets then underwent digestion with 1.0 mg/mL collagenase at room temperature for 6 h, followed by digestion with pronase-E (0.5 mg/mL) for 6 h and trypsin (0.5 mg/mL) for 6 h as described by Paik et al. (28). Another portion of the livers and the second kidney underwent a similar procedure, except they were digested with each enzyme for 24 h at room temperature (40).

**Metabolite Analysis by Radio-TLC.** All samples were immediately analyzed by radio-TLC. The distribution of

**Table 1. TLC and HPLC Retentions of Standard Compounds<sup>a</sup>**

standard compound	$R_f$	$R_t$ (min)	radiochemical purity
<sup>111</sup> In-DTPA-octreotide	0.50	20.60	100%
<sup>111</sup> In-DTPA-D-Phe-OH	0.85	7.05	>98%
<sup>111</sup> In-DTPA-D-Phe-Cys-OH	0.85	14.70	>95%
<sup>111</sup> In-acetate	0.00	4.00 (void) <sup>b</sup>	ND <sup>c</sup>
<sup>111</sup> In-DTPA	1.00	4.00 (void)	ND
<sup>111</sup> In-transferrin	0.00	ND	ND

<sup>a</sup> Radiochemical purity was determined by HPLC integration.

<sup>b</sup> Void indicates that the activity was eluted in the void volume.

<sup>c</sup> ND signifies that no data was collected.

activity was determined by integration of the chromatograms. The kidney supernatants were cospotted with the standards <sup>111</sup>In-DTPA-D-Phe-Cys-OH and <sup>111</sup>In-DTPA-D-Phe-OH and also with <sup>111</sup>In-DTPA and <sup>111</sup>Indium-transferrin for comparison. To further evaluate the metabolites by TLC, centricon filters were used to determine if organ extracts contained protein bound activity. Organ supernatants were passed through a centricon filter with a molecular mass cutoff of 30 000 Da, and the top and bottom fractions were analyzed.

**Metabolite Identification by HPLC.** In separate experiments, liver, kidney, tumor, and adrenal supernatants were analyzed by HPLC at 2, 6, or 8 and 24 h postinjection, and the pancreas supernatant was analyzed at 24 h. Supernatants with low amounts of radioactivity per milliliter were concentrated using a stream of nitrogen. To ensure reproducibility of retention, samples were diluted to less than 5% EtOH and the maximum volume injected was 300  $\mu$ L. To verify the integrity of the standards under our extraction conditions, <sup>111</sup>In-DTPA-D-Phe-Cys-OH and <sup>111</sup>In-DTPA-D-Phe-OH were each added to a kidney, one 2 mL extraction was performed and the supernatant was analyzed by HPLC. To ensure that all activity was accounted for, fractions were collected from the 2 and 6 h analysis of the kidney, counted, and the activity compared to the amount injected. Greater than 90% of the activity was recovered from the column. To demonstrate comigration of the metabolites with each standard, the 6 h kidney supernatant was combined with <sup>111</sup>In-DTPA-D-Phe-Cys-OH and <sup>111</sup>In-DTPA-D-Phe-OH separately and then analyzed by HPLC.

## RESULTS

**Analysis of Standards.** The standards DTPA-D-Phe and DTPA-D-Phe-Cys were analyzed by electron spray mass spectrometry and the expected mass peaks were observed at 541 and 644, respectively. The radiochemical purities of <sup>111</sup>In-DTPA-octreotide, <sup>111</sup>In-DTPA-D-Phe-Cys-OH, and <sup>111</sup>In-DTPA-D-Phe-OH were assessed using reversed-phase HPLC and radio-TLC. Radiochemical purities and chromatographic retentions of the <sup>111</sup>In labeled agents used are listed in Table 1.

**Extraction of <sup>111</sup>In-DTPA-octreotide and Metabolites from Tissues.** Organ blanks were done for every experiment in order to establish the inherent extraction efficiency of <sup>111</sup>In-DTPA-octreotide from the organ matter. This extraction efficiency was then used as a correction factor in determining the percent authentic intact (see Table 3).

Extraction efficiencies for each organ over the time course of an experiment are listed in Table 2. We found that, for the liver and kidney, the extraction efficiencies decreased over time, whereas for the tumor and pancreas, extraction efficiencies were initially lower than the other organs and remained fairly constant. Adrenals exhibited

**Table 2. Extraction Efficiencies Are Listed for Each Organ at the Time Points Studied Postinjection<sup>a</sup>**

time (h)	liver	kidney	tumor	adrenal	pancreas
0	89.3 $\pm$ 9.7	91.6 $\pm$ 3.8	89.0 <sup>b</sup>	89.3 $\pm$ 12.9	99.9 <sup>b</sup>
1	82.5 $\pm$ 7.8	88.0 $\pm$ 1.4	62.0 $\pm$ 5.7	87.1 $\pm$ 13.6	55.8 $\pm$ 0.5
2	68.0 $\pm$ 0.0	84.5 $\pm$ 2.1	65.5 $\pm$ 13.4	86.7 $\pm$ 13.5	31.5 $\pm$ 1.9
4	64.5 $\pm$ 0.7	77.5 $\pm$ 2.1	77.5 $\pm$ 12.0	87.0 $\pm$ 9.4	20.3 $\pm$ 2.6
6	57.5 $\pm$ 7.8	66.5 $\pm$ 5.0	ND	85.4 $\pm$ 7.7	14.4 $\pm$ 6.8
8	ND <sup>c</sup>	ND	51.0 $\pm$ 0.0	ND	ND
21	21.0 <sup>b</sup>	45.0 <sup>b</sup>	53.0 <sup>b</sup>	77.0 <sup>b</sup>	0.0 <sup>b</sup>

<sup>a</sup> Normal organs are from Sprague-Dawley rats, whereas the tumor was excised from lewis rats ( $n = 3$  or 4 unless noted). The data is expressed as: % extraction = cpm in supernatant/(cpm in pellet + cpm in supernatant). <sup>b</sup>  $n = 2$  for these data. <sup>c</sup> ND, no data was collected at this time postinjection.

high extraction efficiencies throughout the experiment, probably due to the small size of the organ and the larger ratio of extraction solvent to tissue used in the extraction procedure.

A digestion procedure was evaluated to maximize the amount of <sup>111</sup>In-labeled intact DTPA-octreotide and metabolites from liver homogenates. Paik et al. determined that this digestion procedure increased their extraction of <sup>111</sup>In-labeled mAb metabolites from 75 to ~95% (28). With <sup>111</sup>In-DTPA-octreotide, it was determined that this procedure did not appreciably increase extraction efficiency, and in all samples, the additional <sup>111</sup>In-labeled agents extracted increased by less than 5%. No further samples were evaluated using this procedure.

**Metabolite Analysis by Radio-TLC.** Following extraction, the organ supernatants were immediately analyzed by radio-TLC. The formation of metabolites with time in the kidney is shown in Figure 3. In all organs, a broad metabolite peak at an  $R_f$  of 0.86 was observed. By TLC, these metabolites were separated from intact <sup>111</sup>In-DTPA-octreotide, which eluted at an  $R_f$  of 0.50. A species was intermittently observed at the origin in small amounts relative to the intact compound. This species did not pass through a centricon filter membrane with a 30 000 MW cutoff, suggesting that <sup>111</sup>In was protein bound.

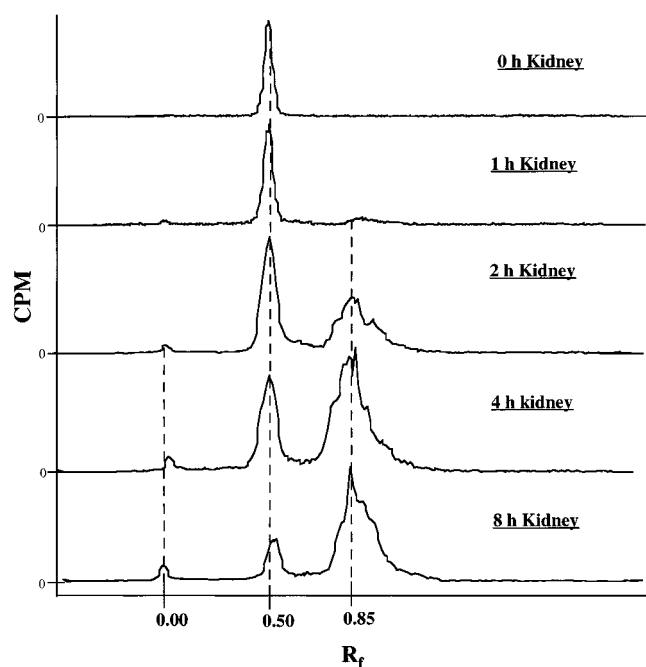
Table 3 shows the percent authentic intact compound present in the organ extracts. These data were calculated from the integration of the radio-thin-layer chromatograms and took into account the unidentified activity left in the pellet, the extraction efficiency of the <sup>111</sup>In-DTPA-octreotide injectate determined from the organ blank experiments and the purity of the injectate. The tumor and pancreas exhibited a similar rate of metabolism with approximately 60% of the extracted activity in the form of intact <sup>111</sup>In-DTPA-octreotide at 1 h postinjection and only 22% at 4 h. In the adrenals, <sup>111</sup>In-DTPA-octreotide appeared to metabolize much more slowly with 99% intact <sup>111</sup>In-DTPA-octreotide at 1 h and 60% intact at 4 h. The liver and kidney exhibited an almost identical rate of metabolism with approximately 80–90% of the activity as intact <sup>111</sup>In-DTPA-octreotide at 1 h and 20% at 4 h. As indicated in Table 3, radioactivity in the blood could only be detected out to 1 h and was present as mostly intact <sup>111</sup>In-DTPA-octreotide. <sup>111</sup>In-DTPA-octreotide was initially excreted mostly intact with 85% of the excreted activity in the form of intact <sup>111</sup>In-DTPA-octreotide at 4 h postinjection. At 12 h postinjection only 23% of the excreted activity was intact compound.

**Metabolite Identification by HPLC.** In separate experiments, HPLC was used to resolve the individual

**Table 3. The Percent of Authentic Intact  $^{111}\text{In}$ -DTPA-Octreotide Determined in Normal Tissues and Tumors Extracted from Sprague–Dawley (Normal Tissues) or Lewis (Tumor) Rats ( $n = 3$  or 4 Unless Noted)<sup>a</sup>**

time (h)	liver	kidney	tumor	adrenals	pancreas	blood	urine
0.0	100.0 <sup>c</sup>	99.9 <sup>c</sup>	100.0 <sup>c</sup>	100.0 <sup>c</sup>	99.9 <sup>c</sup>	88.4 ± 4.6	ND
0.5	ND <sup>b</sup>	ND	ND	ND	ND	87.3 ± 4.6	ND
1.0	78.7 ± 7.3	88.5 ± 1.9	62.4 ± 2.7	99.2 ± 13.3	55.8 ± 0.5	39.9 ± 0.1	100
2.0	46.0 ± 5.3	48.1 ± 4.9	49.0 ± 4.6	75.6 ± 7.8	31.5 ± 1.9	ND	100 <sup>c</sup>
4.0	23.0 ± 1.9	20.4 ± 2.2	23.8 ± 1.0	60.3 ± 12.3	20.3 ± 2.6	ND	85.0 <sup>c</sup>
6.0	17.2 ± 2.1	15.4 ± 6.5	ND	49.3 ± 9.4	14.4 ± 6.8	ND	73.5 <sup>c</sup>
8.0	ND	ND	4.3 ± 0.7	ND	ND	ND	ND
12.0	ND	ND	ND	ND	ND	ND	22.9 <sup>c</sup>
21.0	0.0 <sup>c</sup>	3.3 <sup>c</sup>	0.0 <sup>c</sup>	15.2 <sup>c</sup>	0.0 <sup>c</sup>	ND	ND

<sup>a</sup> The % authentic intact (%AI)  $^{111}\text{In}$ -DTPA-octreotide determined from the integration of the radio-TLCs and calculated as follows: %AI = %P × %I/[1 + (%Pellet/%Super)]%E. %P = purity of injectate determined by TLC; %I = intact  $^{111}\text{In}$ -DTPA-octreotide determined by integration of the TLC; %Pellet/%Super = the ratio of the radioactivity in the pellet and supernatant of the organ blank; %E = the extraction efficiency of the harvested organ after injection of  $^{111}\text{In}$ -DTPA-octreotide. <sup>b</sup> ND signifies that no data was collected at this time postinjection. <sup>c</sup>  $n = 2$ .



**Figure 3.** Radio-TLC time course of metabolite formation in the kidney shown out to 6 h postinjection.  $^{111}\text{In}$ -DTPA-octreotide migrated with an  $R_f$  of 0.50 whereas the metabolites comigrated with an  $R_f$  of 0.85.

metabolites which, when analyzed by radio-TLC, coeluted at  $R_f = 0.86$ . In all organs studied two major metabolites were observed eluting at 7.0 and 14.6 min. To elucidate the identity of these metabolites the 6 h kidney supernatant was co-injected with  $^{111}\text{In}$ -DTPA-D-Phe-Cys-OH and  $^{111}\text{In}$ -DTPA-D-Phe-OH separately as shown in Figure 4. The species eluting at 7.0 min increased in integration area upon co-injection with  $^{111}\text{In}$ -DTPA-D-Phe-OH and the species eluting at 14.6 min increased in area upon co-injection with  $^{111}\text{In}$ -DTPA-D-Phe-Cys-OH. These data strongly suggest that the metabolites of  $^{111}\text{In}$ -DTPA-octreotide were  $^{111}\text{In}$ -DTPA-D-Phe-Cys-OH and  $^{111}\text{In}$ -DTPA-D-Phe-OH.

The HPLC time course for formation of metabolites in the kidney is shown in Figure 5. At 24 h postinjection, no intact  $^{111}\text{In}$ -DTPA-octreotide was observed, 85% of the activity coeluted with  $^{111}\text{In}$ -DTPA-D-Phe-OH, and 7% coeluted with  $^{111}\text{In}$ -DTPA-D-Phe-Cys-OH. In the adrenals at 24 h, 12% of the activity eluted as  $^{111}\text{In}$ -DTPA-octreotide, 28% as  $^{111}\text{In}$ -DTPA-D-Phe-Cys-OH and 60% as  $^{111}\text{In}$ -DTPA-D-Phe-OH. These kidney and adrenal samples were analyzed immediately after organ preparation; however, other samples were stored up to 24 h at

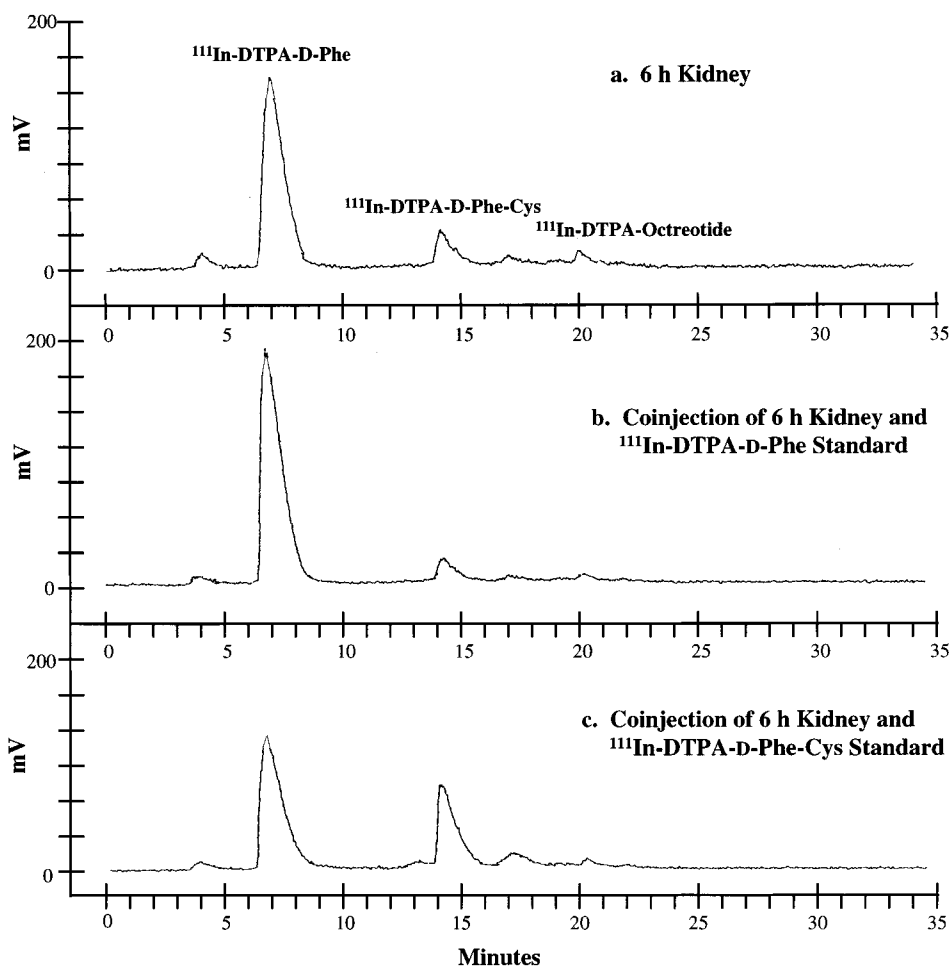
–20 °C prior to analysis. After storage of the supernatants we observed further conversion of  $^{111}\text{In}$ -DTPA-D-Phe-Cys-OH to  $^{111}\text{In}$ -DTPA-D-Phe-OH (as indicated by the differing 6 h kidney chromatograms in Figure 4 versus Figure 5).

## DISCUSSION

To design octreotide radiopharmaceuticals with optimal targeting (high target-to-nontarget ratios of radio-label), the mechanisms by which these agents are internalized, degraded, and released must be understood. In this study, we set out to determine if  $^{111}\text{In}$ -DTPA-octreotide was metabolized in normal and target tissues, to compare the rates of degradation in these organs and to elucidate the identity of the metabolites.

The models used in these studies were normal and tumor-bearing rats. To obtain reproducible results, the methods for performing these *in vivo* studies had to be refined. This first involved maximizing the extraction efficiencies of  $^{111}\text{In}$ -DTPA-octreotide and its metabolites from the selected tissues. In Table 2, the extraction efficiencies are listed for each organ over the time course studied. The same extraction procedure was used each time yet a decrease in extraction efficiencies was observed in the liver and kidney over time postinjection. The extraction efficiencies of the pancreas and tumor were initially lower than the other organs and did not vary much with time. At all times, greater than 75% of the activity was extracted from the adrenals.

The decrease in extraction efficiency with respect to time postinjection could represent the inability to extract an unidentified metabolite from the pellet, the binding of  $^{111}\text{In}$  to intracellular proteins or the inefficient release of internalized metabolites during the extraction procedure. Paik et al. exhaustively extracted  $^{111}\text{In}$ -DTPA-containing metabolites from the liver after injection of a  $^{111}\text{In}$ -DTPA-labeled antibody and obtained a maximum of 75% extraction efficiency (28), whereupon with enzyme digestion of the liver homogenate they obtained greater than 90% extraction efficiency. In our experiments, exhaustive extractions did somewhat increase the activity extracted, but after the first two extractions, we did not observe appreciable increases in amounts of activity. Interestingly, radio-TLC and HPLC showed the same metabolite profile for each individual extraction, indicating that there were no new metabolites extracted in the later extractions. We observed an increase in extraction efficiency upon sonication of the organ samples, indicating the importance of bursting the intracellular vesicles; however, the digestion procedure similar to that described by Paik et al. (28) did not significantly increase the extraction efficiency in our hands.



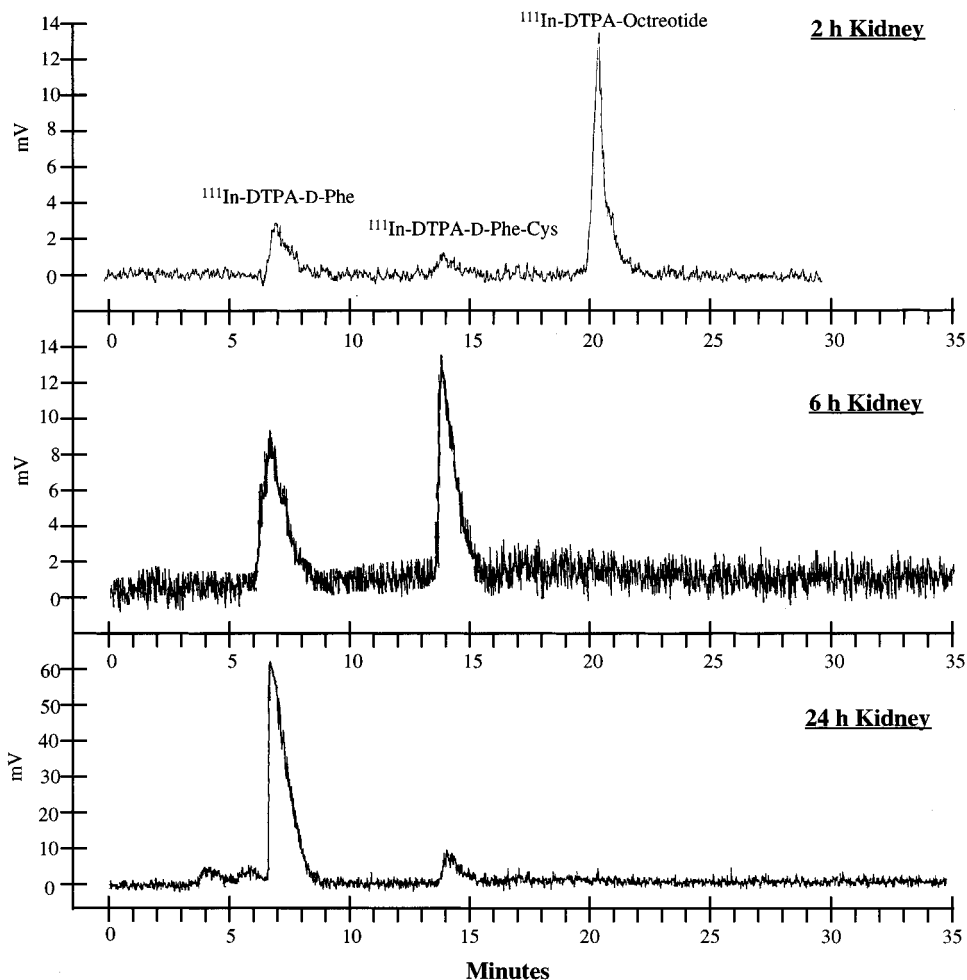
**Figure 4.** HPLC co-injection of the kidney supernatant with the standards <sup>111</sup>In-DTPA-D-Phe-Cys-OH and <sup>111</sup>In-DTPA-D-Phe-OH. (a) In an aliquot of the 6 h kidney supernatant the integrated area of the peak eluting at 7.0 min was  $7 \times 10^6$  mV·s and that of peak eluting at 14.6 min was  $9 \times 10^5$  mV·s. (b) To the same volume aliquot of the 6 h kidney supernatant <sup>111</sup>In-DTPA-D-Phe-OH was added. The integrated area of the peak eluting at 7.0 min increased to  $11 \times 10^6$  mV·s whereas the area of the peak eluting at 14.6 min remained constant at  $9 \times 10^5$  mV·s. (c) <sup>111</sup>In-DTPA-D-Phe-Cys-OH was added to the same volume aliquot of the 6 h kidney. The integrated area of the peak eluting at 7.0 min was equal to that in panel a ( $7 \times 10^6$  mV·s) whereas the area of the peak eluting at 14.6 min increased to  $36 \times 10^6$  mV·s.

A low extraction efficiency may indicate that it is more difficult to extract the metabolites than to extract the intact compound, or it may reflect the possibility that <sup>111</sup>In was bound to insoluble tissue proteins and was therefore not extracted. It is unlikely that the activity in the pellet is intact <sup>111</sup>In-DTPA-octreotide; however, because the identity of this activity is unknown, the percent authentic intact data presented in Table 3 has a degree of error associated with it. It is unlikely that the pellet contains polar or nonpolar smaller molecular weight metabolites, since several different combinations of polar and nonpolar solvents were used in order to increase extraction efficiency. Different combinations of isopropyl alcohol, deionized water, ethanol, acetonitrile, and a variety of buffers (including TEA, HEPES) and detergents (Icon-x, Triton-x) were attempted, and it was determined from these studies that no additional metabolites were extracted. Maximum extraction efficiency was obtained in 20% HEPES in ethanol.

Organ supernatants were analyzed by radio-TLC, and integration of these chromatograms allowed us to compare the rates of metabolism of <sup>111</sup>In-DTPA-octreotide in the various organs studied. As Table 3 indicates, similar rates of metabolism were observed for the tumor, pancreas, liver, and kidney. HPLC analysis provided resolution of the individual metabolites and revealed that in all organs studied the chromatographic retentions of the

two major soluble metabolites were the same. To identify these metabolites two DTPA-amino acid standards were synthesized. <sup>111</sup>In-DTPA-D-Phe-OH was chosen as a standard based on the lysosomal metabolism patterns of other <sup>111</sup>In-DTPA-polypeptides (25, 26, 30, 35). We also analyzed <sup>111</sup>In-DTPA-D-Phe-Cys-OH as a metabolite standard because of the potentially higher stability of the D-Phe-Cys bond (since Phe is a D-amino acid as opposed to the naturally occurring L-amino acids). The chromatographic elutions of the observed metabolites corresponded to those for <sup>111</sup>In-DTPA-D-Phe-Cys-OH and <sup>111</sup>In-DTPA-D-Phe-OH. As shown in Figure 4, co-injection of these standards with the 6 h kidney supernatant confirmed coelution. Identity of the metabolites is not, however, proven by coelution. Future characterization studies involving LC-MS (liquid chromatography-mass spectrometry) of the organ homogenates are planned to confirm the identity of the metabolites of <sup>111</sup>In-DTPA-octreotide.

Although HPLC provided better resolution, one caveat to its use for the study of metabolism is the length of time it takes for each analysis. Due to the time required for sample preparation and analysis, certain samples had to be stored at  $-20$  °C for up to 24 h before analysis. We observed further conversion of <sup>111</sup>In-DTPA-D-Phe-Cys-OH to <sup>111</sup>In-DTPA-D-Phe-OH after 24 h of storage at  $-20$  °C with subsequent thawing at room temperature. This was



**Figure 5.** HPLC time course of metabolite formation in the kidney shown out to 24 h postinjection.  $^{111}\text{In}$ -DTPA-octreotide eluted at 20.5 min,  $^{111}\text{In}$ -DTPA-D-Phe-Cys-OH eluted at 14.6 min and  $^{111}\text{In}$ -DTPA-D-Phe-OH eluted at 7.0 min.

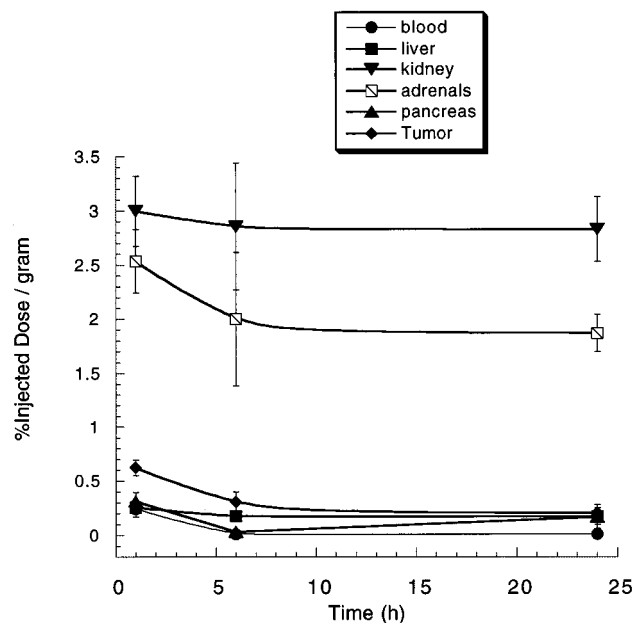
apparent in the 6 h kidney supernatant which initially had 54% of the activity eluting as  $^{111}\text{In}$ -DTPA-D-Phe-Cys-OH and 46% as  $^{111}\text{In}$ -DTPA-D-Phe-OH (Figure 5), yet after 24 h of storage it had only 11% of the activity eluting as  $^{111}\text{In}$ -DTPA-D-Phe-Cys and 84% of the activity as  $^{111}\text{In}$ -DTPA-D-Phe-OH (Figure 4). We were therefore unable to use HPLC as a means for accurately quantifying the distribution of metabolites in the samples.

The results obtained in this study will enable a better understanding of the factors that govern uptake and retention of peptides. We observed less than 25% of the activity in the form of  $^{111}\text{In}$ -DTPA-octreotide in the tumor, pancreas, liver, and kidney at 4 h postinjection. We showed that, out to 21 h postinjection, approximately 50% of the activity in the organs was extractable and contained the metabolites  $^{111}\text{In}$ -DTPA-D-Phe-Cys-OH and  $^{111}\text{In}$ -DTPA-D-Phe-OH. Therefore, it is interesting to note that, in scintigraphic-imaging experiments, which are often carried out at 24 h postinjection, the images most likely represent metabolites remaining in the tissues and not intact  $^{111}\text{In}$ -DTPA-octreotide.

To design octreotide radiopharmaceuticals that will result in enhanced target to nontarget ratios, it is important that the identity of the retained radiolabel is known and the transport mechanisms available to the various organs are taken into account. Breeman et al. demonstrated that  $^{111}\text{In}$ -DTPA-octreotide was taken up in the target organs in less than 20 min postinjection (36). Duncan et al. showed that  $^{111}\text{In}$ -DTPA-octreotide was internalized into the cell and delivered to the lysosomes

within 1 h postinjection (37). A preliminary experiment was carried out to analyze by TLC the kidney lysosomal fraction at 1 h postinjection of  $^{111}\text{In}$ -DTPA-octreotide (Duncan et al., unpublished results). The majority of the activity was in the form of intact  $^{111}\text{In}$ -DTPA-octreotide. These results were in agreement with our studies presented here and indicate that  $^{111}\text{In}$ -DTPA-octreotide was delivered intact to the lysosomes and then degraded.

In biodistribution studies previously carried out by our group, retention of radioactivity was observed in the kidney, liver, and target organs (Figure 6) (15). Duncan et al. showed that at 20 h  $^{111}\text{In}$  was retained in the lysosomes in the kidney, liver, tumor, and pancreas (37). Our data presented here show that, in these organs at 20 h postinjection,  $^{111}\text{In}$ -DTPA-octreotide was almost completely metabolized to  $^{111}\text{In}$ -DTPA-D-Phe-OH and  $^{111}\text{In}$ -DTPA-D-Phe-Cys-OH and other insoluble metabolites. This retention must therefore be dependent on the cellular mechanisms available for transport of these metabolites out of the lysosomes. Although the clearance of activity from the various organs differed, as verified by the biodistribution results, we found the rates of metabolism to be very similar. This implies that the mechanisms by which the degradation products of radiometal-peptide complexes are transported from the cells varies with organ type. Recently, it has been shown that these degradation products are poor substrates for the carrier-mediated transport systems found in lysosomes (26, 30, 37). Although other routes are available, they are slow, and thus, radiometal metabolites ac-



**Figure 6.** Biodistribution of <sup>111</sup>In-DTPA-octreotide in CA20948 tumor-bearing Lewis rats. The data are presented as % injected dose/gram of tissue  $\pm$  standard deviation;  $n = 4$ .

accumulate in the lysosomes of certain organs. Understanding the chemistry of the metabolites may allow us to take advantage of the specialized transmembrane transporters found in the hepatocyte and renal tubular cells that are not present in the target organs, therefore enhancing target to nontarget ratios of radiolabel.

This study along with studies done on the metabolism of other <sup>111</sup>In-DTPA-polypeptides stress the importance of the terminal amino acid (or amino acid to which conjugation occurs) on the fate of the radiolabel. Therefore, one approach to exploiting the different transport mechanisms may involve altering the terminal amino acid (as long as receptor binding is not compromised). Wu et al. observed the formation of two metabolites from an antibody fragment (41). These metabolites were identified as <sup>67</sup>Ga-2-(*p*-SCN-Bz)-NOTA-Met and <sup>67</sup>Ga-2-(*p*-SCN-Bz)NOTA-Lys and it was found that the Met containing metabolite had a significantly shorter residence time in the kidney than did the Lys containing metabolite. We are currently exploring the export mechanisms available to the liver and kidney in hopes of understanding the substrates they transport.

In conclusion, we have shown that <sup>111</sup>In-DTPA-octreotide is metabolized in the blood, liver, kidney, adrenals, pancreas, and tumor and have compared the rate at which metabolism occurs in these tissues. There are no significant differences in the rate of metabolism between target and nontarget tissues, with the exception of slower metabolism in the adrenal glands. The HPLC data indicate that <sup>111</sup>In-DTPA-D-Phe-Cys-OH and <sup>111</sup>In-DTPA-D-Phe-OH are the major extractable metabolites in both the target and nontarget organs; however, there are unknown insoluble metabolites that were not extracted from the tissues. Upon correlation of biodistribution results with the metabolism studies, we conclude that retention of <sup>111</sup>In in the organs studied is likely due to these metabolites. Evaluating other bifunctional chelates or peptides with different N-terminal amino acids may aid in increasing the clearance of these metabolites from tissues. In the design of radiometal-chelate-peptide conjugates that clear normal tissues but are retained in target tissues, we are currently investigating bifunctional

chelates that have specificity for transport systems found in nontarget organs such as liver and kidney, but are absent from target tissues such as tumors.

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