Radiolabelling of Glucose-Tyr^3-octreotate with ^{125}I and Analysis of Its Metabolism in Rats: Comparison with Radiolabelled DOTA-Tyr^3-octreotate

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Abstract. *Background: Somatostatin analogues labelled with radiometals or radiohalogens are useful for the imaging and treatment of somatostatin receptor-containing tumours. In this study, the procedures for the radioiodination of glucose-Tyr^3-octreotate (gluc-Tyr^3-tate) and radiolabelling of DOTA-Tyr^3-octreotate (DOTA-Tyr^3-tate) with ^{111}In, ^{177}Lu and ^{125}I were compared and their metabolism in rats was analyzed. The usefulness of high performance liquid chromatography (HPLC) analysis and instant thin-layer chromatography on silica gel (ITLC-SG) for both radiochemical purity determination and analysis of metabolism in urine was investigated. Materials and Methods: For labelling with radiometals, the formation of a complex with the 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) functionality of the peptide was employed. Radioiodination was performed by the chloramime-T method. The radiochemical purity of radiolabelled peptides and the analyses of rat urine were determined by HPLC and/or ITLC-SG methods. Male Wistar rats were used in the elimination studies. Results: DOTA-Tyr^3-tate was simply radiolabelled with radiometals with high yield and high radiochemical purity. Stopping of the reaction was a critical step for radioiodination, therefore labelling of gluc-Tyr^3-tate and DOTA-Tyr^3-tate with ^{125}I was not so simple and the reaction product had to be purified by preparative HPLC analysis. Whereas ^{111}In-DOTA-Tyr^3-tate and ^{177}Lu-DOTA-Tyr^3-tate were eliminated in rat urine in a practically unchanged form, a significant proportion of metabolites was observed with radioiodinated peptides, particularly at longer time intervals. Conclusion: Labelling of DOTA-Tyr^3-tate with radiometals is simple and the radiochemical purity of prepared compounds is very high, while iodination of the peptides demands purification of the product by preparative HPLC. The analysis of rat urine showed that excretion of radioiodinated peptides included a significant proportion of metabolites.*

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Key Words: Somatostatin peptide analogues, radiolabelling, radiochemical purity, metabolism.

Radiolabelled biomolecules are used in nuclear medicine for both the diagnosis and treatment of many diseases (1, 2). Radiopharmaceuticals with affinity towards specific receptors are mostly derived from biopolymers or their simpler building elements, such as polypeptides, antibodies and oligonucleotides. A large number of peptides have been found which can be used not only for diagnosis, but also for therapy in many pathological cases (1-3). Neuropeptides, especially analogues of somatostatin, are a very important group of such compounds. Receptors of somatostatin are localized on the cell membrane of tumours of endocrine origin, but also are present on other tumours under endocrine control (4-6). Somatostatin, an endogenous tetradecapeptide, acts both as a secretion inhibitor of growth hormone and as an inhibitor of growth of several tumours. Radiolabelled somatostatin is unsuitable for clinical application because of its very short biological half-life, which is why multiple synthetic somatostatin derivatives with a similar bioactive amino acid sequence have been developed (7, 8). DOTA-Tyr^3-octreotate (DOTA-Tyr^3-tate) is a somatostatin analogue with a very high affinity towards the somatostatin receptor subtype2 ($sstr_2$), which is abundant in the majority of endocrine tumours (9). The macrocyclic chelator 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) is able to bind different radiometals (radioindium, radioyttrium, radioactive lanthanides and others), while including tyrosine in the peptide structure allows labelling with radioiodine (10-12). Recent studies have shown that conjugation of glucose to the Tyr^3-octreotate core increased internalization activity in $sstr_2$-

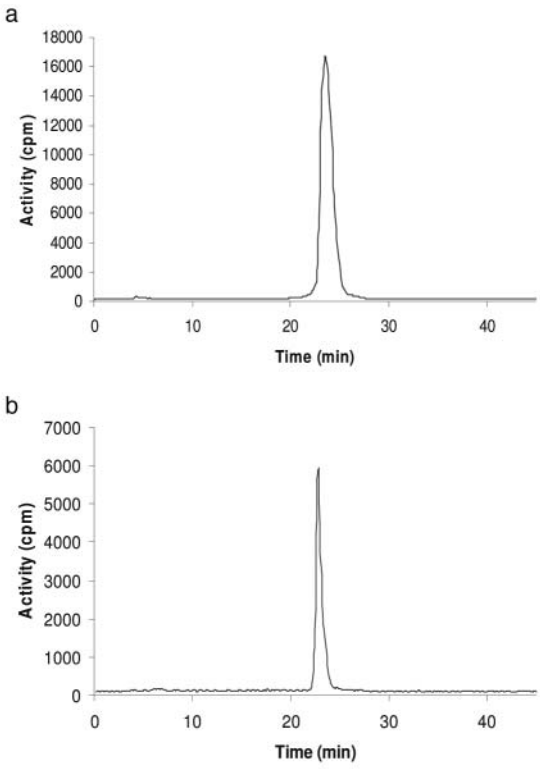

Figure 1. *Examples of HPLC analysis of radiochemical purity. a: ^{111}In-DOTA-Tyr^3-octreotate. b: ^{177}Lu-DOTA-Tyr^3-octreotate.*

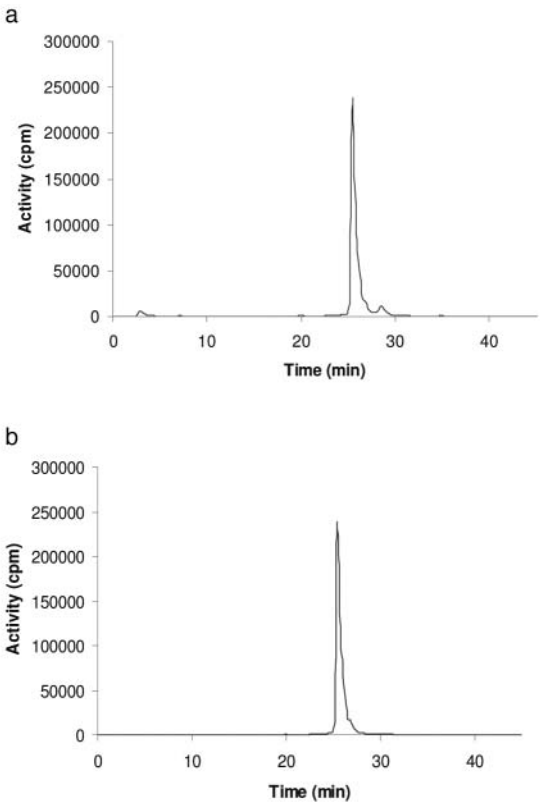

Figure 2. *Examples of HPLC analysis of radiochemical purity. Glucose-^{125}I-Tyr^3-octreotate before (a) and after (b) purification of the reaction product by preparative HPLC. DOTA-^{125}I-Tyr^3-octreotate had a similar profile.*

expressing cell lines (13). The aims of this study were to compare the procedures for the radioiodination of glucose-Tyr^3-octreotate (gluc-Tyr^3-tate) and radiolabelling of DOTA-Tyr^3-tate with ^{111}In (used for diagnosis), ^{177}Lu (used for cancer treatment) and ^{125}I (used for diagnostics and therapy) and to evaluate the methods of analysis of the labelled agents and their metabolites in urine.

Materials and Methods

Materials and reagents. The gluc-Tyr^3-tate was synthesized at Duke University Medical Center as described previously (13). The DOTA-Tyr^3-tate was prepared by piCHEM Research and Development, Graz, Austria. All other chemicals used were purchased from commercial sources. For high performance liquid chromatography (HPLC) analysis a Pharmacia-LKB system with Gradient Master GP 962 (Institute of Organic Chemistry and Biochemistry, Prague, Czech Republic) equipped with a LichroCART 125-4 or 250-4 HPLC Cartridge Lichrospher RP C18e, 5 μm (Merck, Darmstadt, Germany) and with a radioactivity monitoring analyzer was used. An automatic gamma counter (1480 Wizard™ 3", Wallac, Finland) was used for counting the fractions on HPLC analysis of the rat urine. Instant thin-layer chromatography on silica gel (ITLC-SG) was performed on commercial silica gel impregnated glass fiber sheets (Gelman Sciences, Michigan, USA). Detection of the ITLC-SG strips was carried out by a TLC-analyzer (Raytest, Straubenhardt, Germany).

Table I. *Radioactivity elimination by urine after radiolabelled gluc-Tyr^3-tate and DOTA-Tyr^3-tate administration to rats (% of administered dose).*

Agent	0-2 h	2-24 h
^{111}In-DOTA-Tyr^3-tate	60.5±12.2	7.0±1.2
^{177}Lu-DOTA-Tyr^3-tate	45.8±10.2	7.7±2.0
DOTA-^{125}I-Tyr^3-tate	39.0±20.7	19.6±2.8
gluc-^{125}I-Tyr^3-tate	21.9±11.5	36.6±5.1

Preparation of ^{111}In-DOTA-Tyr^3-tate. Two hundred μl of 0.4 M acetate buffer (pH=5) with 0.24 M gentisic acid, 10 μg DOTA-Tyr^3-tate in 10 μl of H_2O and 0.5 mCi $^{111}InCl_3$ in 0.04 M HCl were successively added to a vial. The solution was well mixed and then incubated at 90-95˚C for 25 min.

Preparation of ^{177}Lu-DOTA-Tyr^3-tate. Two hundred μl of 0.4 M acetate buffer (pH=5) with 0.24 M gentisic acid, 10 μg DOTA-Tyr^3-tate in 10 μl of H_2O and 1 mCi $^{177}LuCl_3$ in 0.05 M HCl were successively added to a vial. The solution was well mixed and then incubated at 90-95˚C for 25 min.

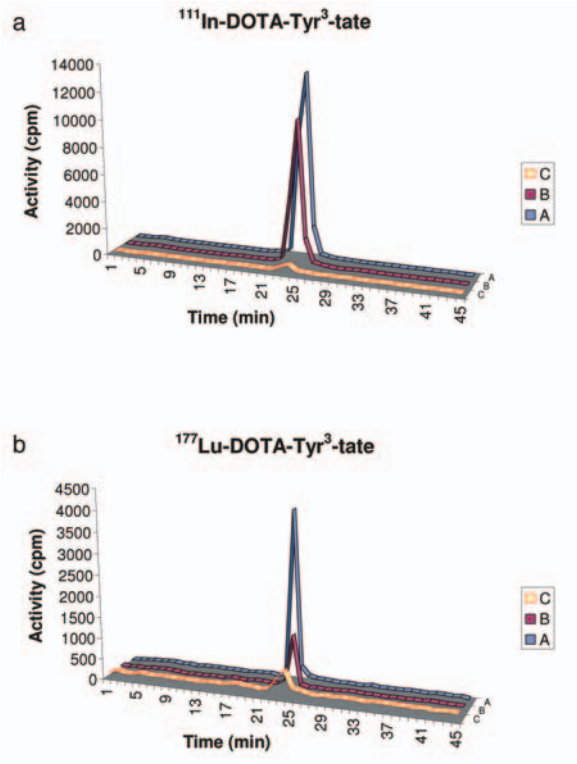

Figure 3. *Examples of HPLC analysis. a: diluted standard of ^{111}In-DOTA-Tyr^3-tate (A), rat urine 2 hours (B) and 24 hours (C) after injection. b: diluted standard of ^{177}Lu-DOTA-Tyr^3-tate (A), rat urine 2 hours (B) and 24 hours (C) after injection.*

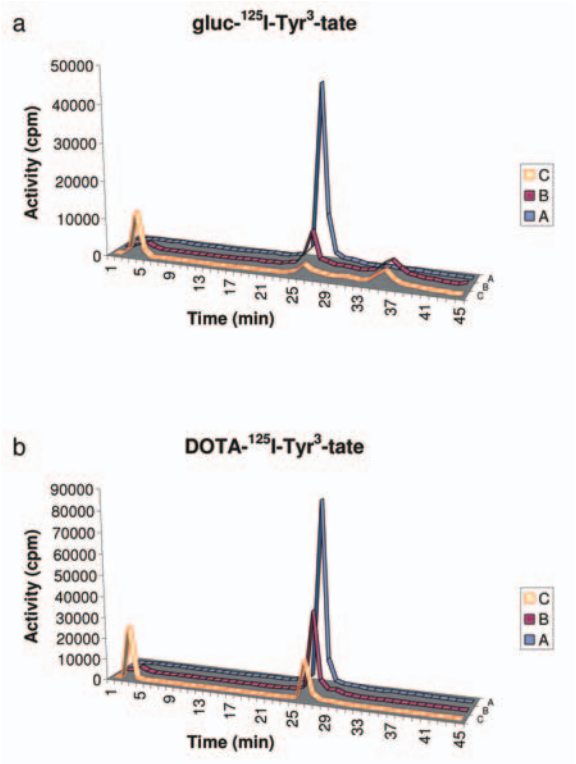

Figure 4. *Examples of HPLC analysis. a: diluted standard of gluc-^{125}I-Tyr^3-tate (A), rat urine 2 hours (B) and 24 hours (C) after injection. b: diluted standard of DOTA-^{125}I-Tyr^3-tate (A), rat urine 2 hours (B) and 24 hours (C) after injection.*

Preparation of gluc-^{125}I-Tyr^3-tate and DOTA-^{125}I-Tyr^3-tate. Fifty μl of 0.05 M phosphate buffer (pH=7.4), 5 μg gluc-Tyr^3-tate or DOTA-Tyr^3-tate in 5 μl of 0.05M acetic acid and 0.4 mCi Na^{125}I were mixed and then 2.5 μg of chloramine T in 2.5 μl of the same 0.05 M phosphate buffer (pH=7.4) was added. After 60 sec reaction at room temperature, the mixture was diluted by the addition of 40 μl of ethanol and after that, the solution was injected onto the HPLC system.

HPLC analysis. The radiochemical purity of the peptides labelled with radiometals was determined by reverse phase HPLC analysis using a LichroCART 125-4 HPLC Cartridge Lichrospher RP C18e, 5 μm and gradient elution with 0.1% trifluoracetic acid as mobile phase A and acetonitrile as mobile phase B. The gradient sequence was 0-5 min 0% B, 5-25 min 0-30% B, 25-30 min 30% B, 30-35 min 30-100% B, 35-40 min 100% B and 40-45 min 100-0% B. The flow rate was 0.5 ml/min.

For the radioiodinated peptides, reverse phase HPLC analysis was conducted on a LichroCART 250-4 HPLC Cartridge Lichrospher RP C18e, 5 μm. Gradient elution with 0.1 M NaCl as mobile phase A and 95% ethanol as mobile phase B was employed for purification of the product. The gradient was 0-10 min 0% B, 10-20 min 0-60% B, 20-30 min 60% B, 30-35 min 60-100% B, 35-40 min 100% B and 40-45 min 100-0% B at a flow rate of 1 ml/min. In addition to detecting radioactivity on line, fractions were taken at 1 minute intervals to analyze radioactivity at the peaks. The mobile phase of the fractions containing gluc-Tyr^3-^{125}I-tate or DOTA-Tyr^3-^{125}I-tate was evaporated at 37°C under a gentle stream of nitrogen and the residue was diluted with saline.

The analysis of rat urine after the injection of the labelled peptides was performed under the same conditions, except that a new column of the same size was used and fractions were taken at 1 minute intervals. These fractions were analyzed on the automatic gamma counter.

ITLC-SG analysis. ITLC-SG analysis was used for the rapid evaluation of the purity of peptides labelled by ^{111}In and ^{177}Lu. ITLC-SG chromatography was performed on silica gel impregnated glass fiber sheets with 10% ammonium acetate and methanol 1:1 as the eluent. In this system, the labelled peptide moves with the solvent front and unbound radiometal remains at the start of the chromatogram. A 1 μl aliquot of labelled peptide was evaluated on each ITLC-SG strip. After elution, the ITLC-SG strips were counted by the TLC-analyzer.

Biological experiments. Male Wistar rats weighing 200-250 g (each experimental group consisted of four animals) were used in the elimination studies and 0.2 ml of radiopharmaceutical (0.8-1 μg of the peptide per kg of body weight) was injected into the tail vein. Each rat was placed in a separate glass metabolic cage. The urine

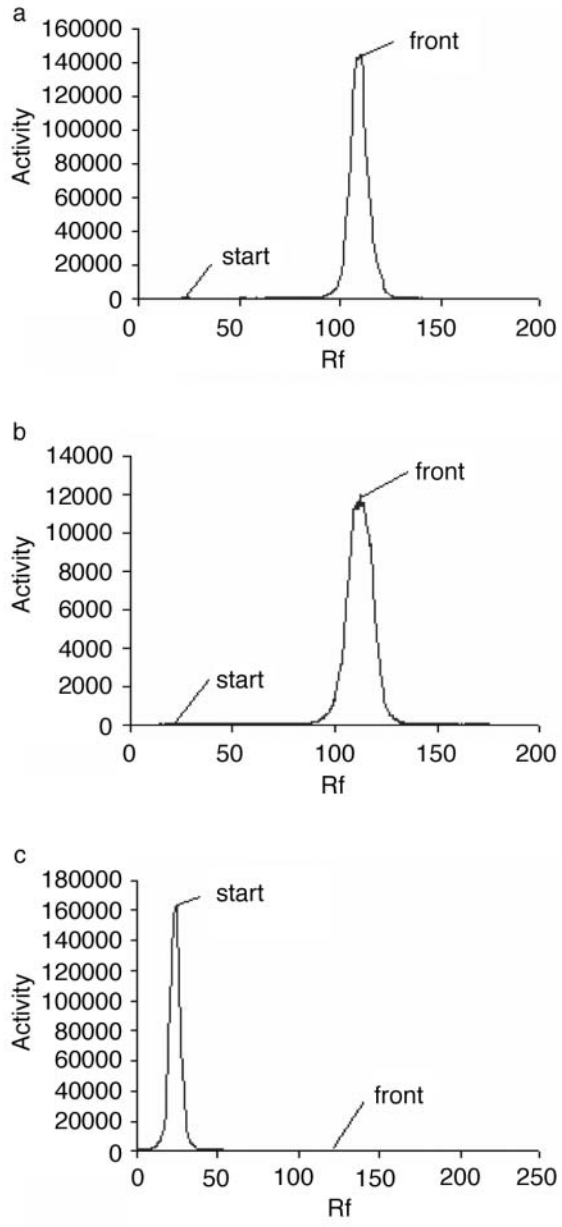

Figure 5. *Examples of ITLC-SG analysis. a: ^{111}In-DOTA-Tyr^3-tate, b: ^{177}Lu-DOTA-Tyr^3-tate, and c: free radiometal (^{111}In, ^{177}Lu).*

was collected 2 h and 24 h after the injection. Handling (immobilization) was used for emptying the urinary bladders of the animals at the above mentioned intervals. All urine eliminated in time intervals 0-2 h and 2-24 h was used for subsequent analysis.

All animal experiments were approved by the Ethics Commission of the Faculty of Pharmacy, Charles University.

Plasma protein binding. The binding of radiolabelled peptides to rat plasma was determined by equilibrium dialysis at 37˚C (14). Plasma was obtained by centrifugation of heparinized rat blood. 0.5 ml of plasma was dialysed against the same volume of saline buffered with 0.01 mol/l phosphate (pH=7.4) with the peptide at the initial concentration of 1 ng/ml for 16 hours.

Table II. *Free fraction of the peptides in rat plasma determined by equilibrium dialysis at 37˚C.*

Agent	Free fraction in rat plasma (%)
^{111}In-DOTA-Tyr^3-tate	77.3±1.0
^{177}Lu-DOTA-Tyr^3-tate	82.5±1.5
DOTA-^{125}I-Tyr^3-tate	58.6±7.1
gluc-^{125}I-Tyr^3-tate	44.4±1.5

Statistical analysis. Statistical analysis was carried out with Student's unpaired *t*-test. Statistical significance was set at $p<0.05$.

Results

Radiochemical purity of labelled peptides. DOTA-Tyr^3-tate was labelled easily with ^{111}In and ^{177}Lu with high radiochemical purity (more than 98%, Figures 1a and b). Labelling of gluc-Tyr^3-tate and DOTA-Tyr^3-tate by oxidative iodination (^{125}I) was simple, but a problematic aspect was the termination of the reaction, because the reducing agents used routinely to accomplish this may also break down the disulfide bridge of the peptide. For that reason, the reaction product after radioiodination was purified by preparative HPLC before its use (Figure 2a and b). The HPLC purification method was able to separate various forms of the radioiodinated peptide.

Elimination of radioactivity via the urine after intravenous administration to rats. The excretion of radioactivity in the urine during the time intervals 0-2 h and 2-24 h after administration of the labelled peptides is presented in Table I. Urinary excretion was the main elimination pathway for these radiopharmaceuticals and renal excretion of radioactivity was relatively rapid.

The proportion of radioactivity excreted by the urine in the time interval 0-2 h after gluc-^{125}I-Tyr^3-tate administration was significantly lower that those for the other radiometal-labelled peptides. No significant differences in cumulative radioactivity elimination between the agents under study in the time interval 0-24 h were found, however.

HPLC analysis of rat urine. HPLC analysis of the rat urine 2 hours and 24 hours after ^{111}In-DOTA-Tyr^3-tate and ^{177}Lu-DOTA-Tyr^3-tate injection indicated that elimination of the parent peptides was the predominant process. In contrast, a significant proportion of the radioactivity present in the rat urine 2 hours and 24 hours after gluc-^{125}I-Tyr^3-tate and DOTA-^{125}I-Tyr^3-tate injection was determined to be in the form of metabolites.

Substantial differences in the proportion of metabolites found in the urine between peptides labelled with ^{125}I and those labelled with radiometals are shown in Figures 3 and 4. Whereas only traces of metabolites after application of radiometal-labelled compounds were detected in the urine, after DOTA-^{125}I-Tyr^3-tate administration, up to 50% of the eliminated radioactivity was in the form of degradation products. In the case of gluc-^{125}I-Tyr^3-tate, most of the eliminated radioactivity (65-75%) in urine was found as breakdown products.

ITLC-SG analysis. ITLC-SG analysis showed very high purity of DOTA-Tyr^3-tate labelled with radiometals. The radiochemical purity determined by this method was greater than 98%, identical to the results measured by HPLC (Figure 5).

Plasma protein binding. The reversible binding of the four radiolabelled peptides to rat plasma proteins was evaluated by equilibrium dialysis. The free peptide fractions are compared in Table II. The free fractions for the two radiometal labelled peptide conjugates were similar and significantly higher ($p<0.05$) that those for either of the radioiodinated peptides. Furthermore, the free fraction for the radioiodinated peptide containing the more polar DOTA prosthetic group was significantly higher than that observed for the glycated peptide conjugate.

Discussion

HPLC is probably the most accurate and sensitive method for an analysis of the purity of radiolabeled octreotate analogues. This method is, however, frequently not available for routine analysis of radiolabelled receptor specific peptides in departments of nuclear medicine, particularly in countries such as the Czech Republic. For this reason, ITLC-SG analysis is usually used as a quicker and simpler alternative to HPLC. In the present study ITLC-SG with HPLC of the four labelled peptides was compared. ITLC-SG analysis showed very high purity of the DOTA-Tyr^3-tate labelled with radiometals. The radiochemical purity was higher than 98%, identical to the results obtained by HPLC analysis. On the other hand, ITLC-SG analysis of the radioiodinated peptides would not be a very useful alternative to HPLC because of its inability to separate labelled peptide from chemically similar byproducts.

In evaluating the renal excretion of the 4 labelled peptides, it was found that the iodinated peptides were eliminated into the urine more slowly than the peptides labelled with radiometals and the gluc-^{125}I-Tyr^3-tate was the slowest. This finding was in agreement with the higher lipophilicity of iodinated compounds, gluc-^{125}I-Tyr^3-tate being the most lipophilic. Another consequence of higher lipophilicity is higher plasma protein binding of the compound, and our results were again consistent with this. Because of their molecular size, the four peptides under study are thought to be eliminated from the blood predominantly by glomerular filtration in the kidney. Considering that the renal elimination rate is a product of the glomerular filtration rate and the free drug fraction in the plasma, higher plasma binding (lower free fraction) results in slower renal elimination rate of the agent.

The difference in the proportion of activity present as metabolites in urine between iodinated peptides and DOTA-Tyr^3-tate labelled with radiometals probably reflected the different handling of the peptides in the somatostatin receptor-rich organs and in the kidney. In the tissues with a high density of somatostatin receptors, labelled peptides are internalized by somatostatin receptor-mediated endocytosis. Also in the kidney, after glomerular filtration, the peptides would have been partly internalized by megalin/cubillin-mediated endocytosis in renal tubules (15, 16). In both types of tissues, the agents would have been subsequently transferred into the lysosomes, wherein they would have been degraded by proteolytic enzymes (17). The degradation products of iodinated DOTA-Tyr^3-tate (free iodide and/or iodinated tyrosine and/or other iodinated fragments) were externalized from somatostatin receptor-rich tissues and from the kidney and finally eliminated into urine, whereas the breakdown products of DOTA-Tyr^3-tate labelled with radiometals are known to persist in receptor-rich tissues for a long time (18).

Conclusion

Labelling of DOTA-Tyr^3-tate with radiometals is simple and the radiochemical purity of the labelled products is very high, while iodination of gluc-Tyr^3-octreotate and DOTA-Tyr^3-tate with ^{125}I is more complicated. Radiochemical purity determination by ITLC-SG was comparable to HPLC analysis for radiometal-labelling, but ITLC-SG analysis of radioiodinated peptides is not useful because of the limited ability to separate the desired labelled peptide from other labelled species. ^{111}In-DOTA-Tyr^3-tate and ^{177}Lu-DOTA-Tyr^3-tate were eliminated by rat urine mainly in an unchanged form, but with iodinated gluc-Tyr^3-tate and DOTA-Tyr^3-tate, a significant proportion of metabolites (iodide and/or iodinated fragments of the peptide) was observed.

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