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Biodistribution and catabolism of ^{18}F-labelled isopeptide N$^{\varepsilon}$-(γ-glutamyl)-L-lysine

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Summary. Isopeptide bonds between the ε-amino group of lysine and the γ-carboxamide group of glutamine are formed during strong heating of pure proteins or, more important, by enzymatic reaction mediated by transglutaminases. Despite the wide use of a microbial transglutaminase in food biotechnology, up to now little is known about the metabolic fate of the isopeptide N$^{\varepsilon}$-(γ-glutamyl)-L-lysine. In the present study, N-succinimidyl-4-[^{18}F]fluorobenzoate was used to modify N$^{\varepsilon}$-(γ-glutamyl)-L-lysine at each of its two α-amino groups, resulting in the 4-[^{18}F]fluorobenzoylated derivatives, for which biodistribution, catabolism, and elimination were investigated in male Wistar rats. A significant different biochemical behavior of the two labelled isopeptides was observed in terms of *in vitro* stability, *in vivo* metabolism as well as biodistribution. The results suggest that the metabolic fate of isopeptides is likely to be dependent on how they are reabsorbed – free or peptide bound.

Keywords: Transglutaminase – N$^{\varepsilon}$-(γ-glutamyl)-L-lysine – Crosslinking – Positron emission tomography

1 Introduction

Transglutaminases (EC 2.3.2.13) are enzymes that belong to the aminoacyltransferases. They are widely distributed in most tissues and body fluids such as blood, epidermis, liver, prostate, and hair follicles. The enzymes, which generally are Ca^{2+}-dependent, form isopeptide bonds between the γ-carboxamide group of glutamine and various primary amines, involving the glutamine residue as the acyl donor and the primary amines as acyl acceptors. The most common acyl acceptor is the ε-amino group of lysine (Griffin et al., 2002). Proteins can be crosslinked by such isopeptide bonds intramolecularly as well as intermolecularly. The crosslinking of proteins usually results in high molecular products which are very resistant to proteolytic degradation and mechanical stress. Treatment of food proteins with transglutaminases has a particular influence on texture by improving gel formation and changing solubility or water binding properties. For applications in food technology, a Ca^{2+}-independent transglutaminase from *Streptoverticillium* sp. is used (Nielsen, 1995). This microbial transglutaminase (MTG) has the advantage of a low-cost availability. In contrast to eukaryotic transglutaminases, MTG is of higher stability and exhibits a lower specificity, which enables applications to a multitude of proteins. MTG is widely used in food technology such as dairy, meat, and bakery industries (Nielsen, 1995).

Up to now, little is known about the metabolic fate of the isopeptides formed by MTG in foods. Waibel and Carpenter reported that the isopeptide N$^{\varepsilon}$-(γ-glutamyl)-L-lysine could almost completely replace L-lysine in the alimentation of growing rats and chickens (Waibel and Carpenter, 1972). Raczynski and coworkers had shown in *in vitro* studies with ^{3}H-labelled N$^{\varepsilon}$-(γ-glutamyl)-L-lysine that there is no cleavage of the isopeptide bond in the digestive tract, and that the isopeptides are absorbed as such (Raczynski et al., 1975). Furthermore, Finot and colleagues examined different α-N- and ε-N-substituted derivatives of lysine for its bioavailability (Finot et al., 1978). For this purpose, N$^{\varepsilon}$-(γ-glutamyl)-L-lysine and L-lysine were labelled with ^{14}C. A delayed bioavailability of such labelled N$^{\varepsilon}$-(γ-glutamyl)-L-lysine relative to labelled L-lysine was observed. This was traced back to the fact that N$^{\varepsilon}$-(γ-glutamyl)-L-lysine is absorbed unchanged in the small intestine and is hydrolyzed by a different organ, most likely by the kidneys. Yasumoto and Suzuki also showed that the isopeptide bond is not cleaved by peptidases of the intestinal mucosa (Yasumoto and Suzuki,

1990). For ^{14}C-labelled isopeptides, the authors observed that more than 90% of administered isopeptide were absorbed in the gut. Furthermore, a release of free L-lysine from N$^{\varepsilon}$-(γ-glutamyl)-L-lysine in kidney homogenates was found. This decomposition of isopeptides was explained by the activity of the enzyme γ-glutamylcyclotransferase (EC 2.3.2.4) which is found in the kidneys. The cleavage of the isopeptide bond caused by this enzyme results in the formation of free L-lysine and 5-oxoproline, which can be further metabolized to glutamic acid by 5-oxoprolinase. Furthermore, Seguro and colleagues reported on the liberation of L-lysine from N$^{\varepsilon}$-(γ-glutamyl)-L-lysine by the enzyme γ-glutamyltranspeptidase (EC 2.3.2.2), occurring both in the kidneys and the intestine (Seguro et al., 1996). Moreover, hydrolysis of the isopeptide bond by transglutaminase was examined by Parameswaran and coworkers (Parameswaran et al., 1997), who described the impact of the coagulation factor XIIIa, cytosolic transglutaminases from human red blood cells and from guinea pig liver on isopeptides. All three enzymes showed isopeptidase activities.

However, detailed mechanisms of isopeptide metabolism *in vivo* are still unclear. Therefore, biodistribution, metabolism, and elimination of ^{18}F-labelled isopeptide N$^{\varepsilon}$-(γ-glutamyl)-L-lysine were investigated in the present study. Based on a suitable radiolabelling method, which was previously published by our group (Wuest et al., 2003), [^{18}F]fluorobenzoylated derivatives of N$^{\varepsilon}$-(γ-glutamyl)-L-lysine were synthesized and the ^{18}F-labelled isopeptide derivatives were characterized in different *in vitro* and *in vivo* investigations in terms of stability and biodistribution.

2 Materials and methods

2.1 General

All chemicals were reagent grade obtained from commercial suppliers and used without further purification. N-succinimidyl-4-[^{18}F]fluorobenzoate ([^{18}F]SFB) was prepared according to Wuest and coworkers (Wuest et al., 2003). N-succinimidyl-4-[^{19}F]fluorobenzoate ([^{19}F]SFB) was prepared according to Wester and colleagues (Wester et al., 1996). Reactions using microwave activation were performed with a MICROWELL 10 oven (Labwell AB, Uppsala, Sweden). For radio-TLC detection (Merck silica gel, n-butanol/water/acetic acid 4/1/1 = v/v/v) a BAS 2000 scanner (FUJIX, Tokyo, Japan) was used.

2.2 Radiolabelling of isopeptide N$^{\varepsilon}$-(γ-glutamyl)-L-lysine

The radiolabelling of N$^{\varepsilon}$-(γ-glutamyl)-L-lysine was carried out using 2 mg of the peptide in 800 μL Kolthoff's buffer pH 8.6 consisting of 0.1 M potassium dihydrogen phosphate solution and 0.05 M disodium tetraborate decahydrate solution and no-carrier-added (n.c.a.) [^{18}F]SFB solved in 200 μL acetonitrile. The reaction mixture was kept at 22°C for 20 min. After addition of 10 μL of trifluoroacetic acid purification was accomplished by semi-preparative C-18 reversed phase chromatography with a Merck LiChroCart 250-10 column using a step gradient. Solvent A comprised water with 0.1% trifluoroacetic acid (TFA), and solvent B comprised acetonitrile with 0.1% TFA: 0–10 min 20% B, 10–30 min 80% B. Fraction **1** ($t_R = 12.8$ min) and fraction **2** ($t_R = 14.4$ min) of labelled isopeptide were collected. As reference substances, the respective non-radioactive [^{19}F]fluorobenzoylated peptides were prepared and identified by electrospray mass spectrometry (Quattro-LC, Micromass).

2.3 Radiolabelling of L-lysine

For synthesis of α-[^{18}F]fluorobenzoylated lysine, 2 mg of H-Lys(Boc)-OH was dissolved in 800 μL Kolthoff's buffer pH 8.4. After addition of n.c.a. [^{18}F]SFB solved in 200 μL acetonitrile, the reaction mixture was kept at 22°C for 20 min. The reaction was performed in a conical vial suitable for microwave activation. Then, 300 μL of 5 N hydrochloric acid was added, the vial was sealed and heated by microwave activation (100 W, 2 min). Purification was accomplished according to section 2.2. The fraction containing labelled lysine **3** ($t_R = 13.8$ min) was collected.

2.4 Radiolabelling of glutamic acid

Glutamic acid was dissolved in 800 μL Kolthoff's buffer pH 8.4 and n.c.a. [^{18}F]SFB, dissolved in 200 μL acetonitrile, was added. The reaction mixture was kept at 22°C for 20 min. Purification was accomplished according to section 2.2. The fraction containing labelled glutamic acid **4** ($t_R = 14.0$ min) was collected.

2.5 In vitro stability of the [^{18}F]fluorobenzoylated isopeptides

For investigation of the *in vitro* stability of the [^{18}F]fluorobenzoylated isopeptides **1** and **2**, tissue homogenates of kidney, liver, pancreas and small intestine from male Wistar rats were used. For this purpose the rats were sacrificed under ether anesthesia, the organs were taken and homogenized using ice cold 0.01 M phosphate buffered saline (PBS) (1/9 = v/v). Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce, IL, USA) with bovine serum albumin as a standard. A protein content of 4.1 g/L was adjusted. For incubation experiments, 1 ml of each homogenate was applied. To each of them 20 μL of labelled isopeptide fraction **1** or fraction **2**, respectively, was added. This volume contained a radioactivity of 3–5 MBq. The mixtures were kept at 37°C. After 5, 15, 30, 60, and 90 min aliquots of 20 μL were taken from the mixture. Protein was precipitated with 40 μL of a solution containing trifluoroacetic acid/methanol/water (5/45/50 = v/v/v). The supernatant was applied for analytic examination, which was performed by thin layer chromatography with radioactivity detection (radio-TLC). Furthermore, analogous investigations were made with tissue homogenates which were heated for 5 min at 80°C to examine if a potential conversion of the labelling products **1** and **2** is actually caused by enzymatic action. Moreover, a kidney homogenate was separated into a membranous and cytosolic fraction. For this purpose, rat kidneys were perfused with 0.01 M PBS and then dissected. Kidneys were homogenized under ice cooling 1:7 with 10 mM Tris/HCl (pH 7.4) containing 0.25 M sucrose, 1 mM EDTA, 0.1 mM PMSF, 0.1 mM benzamidine, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, and 1 mg/mL aprotinin in a glass homogenizer. After sedimentation of the nuclei and mitochondria at 12,000 × g for 10 min, the post-mitochondrial supernatant was centrifuged at 100,000 × g for 20 min. From the supernatant cytosolic fraction aliquots were taken and frozen at −80°C until used. The resulting pellet was resuspended and recentrifuged at 12,000 × g to remove mitochondrial contamination. The supernatant was then centrifuged at 100,000 × g for 20 min. All centrifugation steps were carried out at 4°C. The final crude membrane pellet was resuspended in 20 mM Tris/HCl, pH 7.4, and was frozen at −80°C until used. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce, IL, USA) with bovine serum albumin as a standard. Again a

protein content of 4.1 g/L was adjusted. Subsequently, 3 MBq of compound **2** in a 20 μL volume was incubated with both membranous and cytosolic fraction. Aliquots were taken and proceeded as described.

2.6 Biodistribution in Wistar rats

2.6.1 Animals

The animal facilities and the experiments were approved according to the German Regulations for Animal Welfare. The protocol was approved by the local Ethical Committee for Animal Experiments. Biodistribution and PET experiments were performed in male Wistar rats (aged 5–6 weeks; 160–170 g).

2.6.2 Biodistribution studies

2–3 MBq of the [^{18}F]fluorobenzoylated compound **1**, **2**, **3** or **4** (radiochemical purity $\geq$95%) were injected into the tail vein of the animals under a light ether anesthesia. The injection volume was 0.5 mL. At 5 min p.i. and 60 min p.i., respectively, the animals were sacrificed by heart puncture under ether anesthesia. The organs and tissues of interest were rapidly excised, weighed, and the radioactivity was determined (Cobra II gamma counter, Canberra-Packard, Meriden, CT, USA). The associated radioactivity in organs and tissues was calculated as the percentage of the injected dose per organ (%ID/organ).

2.6.3 PET data acquisition and imaging studies

Dynamic PET studies were performed with a dedicated PET scanner for small animals (microPET P4, CTI Concorde Microsystems, Knoxville, TN, USA). For imaging studies, animals were anesthetized with urethane (1.3 g/kg body weight) and catheters were placed into the right external jugular vein or, alternatively, into the femoral vein. The animals under urethane anesthesia were then positioned and immobilized supine with their medial axis parallel to the axial axis of the scanner with thorax and abdominal region (organs of interest: heart, liver, kidneys, bladder) in the center of field of view. In a typical experiment, approximately 15 MBq of the [^{18}F]fluorobenzoylated compound **1**, **2**, **3** or **4** were administered within 15 seconds in a 0.5 mL volume. Simultaneously with tracer injection, dynamic PET scanning was started for 45–75 min using the following time intervals (frames) for sinogram generation: 12 $\times$ 10 s, 6 $\times$ 30 s, 5 $\times$ 300 s, 3 $\times$ 600 s, and 1 $\times$ 900 s. Sinogram generation and image reconstruction followed the protocol given by us elsewhere (Pietzsch et al., 2005).

2.6.4 *In vivo* stability

The *in vivo* stability of the compounds **1**, **2**, **3** and **4** was analyzed using rat arterial blood samples and urine at 5 min and 60 min p.i. Aliquots of the arterial blood were removed and immediately centrifuged. The arterial plasma samples and the urine were mixed with twice the volume of water/methanol/trifluoroacetic acid (50/45/5 = v/v/v) to precipitate the protein. After centrifugation at 10,000 $\times$ g for 2 min the supernatant was used for analytical HPLC. Furthermore, metabolites in kidneys were analyzed. For this purpose a 10% homogenate from kidneys was prepared in isotonic saline solution. After addition of water/methanol/trifluoroacetic acid (50/45/5 = v/v/v) and centrifugation the supernatant was used for the HPLC analysis.

HPLC analysis was performed using a Zorbax 300 SB-C18 (9.4 $\times$ 250 mm, 5 μm)-column. Typically, a 5–20 μl sample was injected. The compounds were separated using gradient conditions and a flow rate of 2 ml/min. Solvent A comprised water with 0.05% trifluoroacetic acid (TFA), and solvent B comprised acetonitrile with 0.04% TFA. The gradient contained the following steps: 0 min 10% B, 10 min 20% B, 15–20 min 80% B. For UV-detection the wavelength of 214 nm was used. Radioactivity signal was detected by a radio-chromatography detector (Canberra-Packard, Germany).

3 Results

3.1 Labelling results

The [^{18}F]fluorobenzoylated compounds were synthesized using no-carrier-added N-succinimidyl-4-[^{18}F]fluorobenzoate ([^{18}F]SFB) at a specific radioactivity of 5–15 GBq/μmol. The coupling reaction of isopeptide N$^{\varepsilon}$-(γ-glutamyl)-L-lysine with [^{18}F]SFB resulted in two different labelling products **1** and **2** due to the presence of two primary amino groups in the isopeptide (Fig. 1). Furthermore, the [^{18}F]fluorobenzoylated derivatives of L-lysine and glutamic acid were prepared because these compounds might result as potential metabolites when the isopeptide bond of the [^{18}F]fluorobenzoylated isopeptides **1** and **2** is cleaved (Figs. 2 and 3). The decay-corrected radiochemical yields were 21 to 25% for both **1** and **2**, 39 to 45% for **3** and 48 to 52% for **4**. All compounds were obtained in radiochemical purity >95%. Starting from [^{18}F]SFB, all labelled products could be synthesized within 50 min including HPLC purification.

3.2 In vitro stability of the [^{18}F]fluorobenzoylated isopeptides

During incubation with the tissue homogenates and subsequent separation and quantification via thin layer chro-

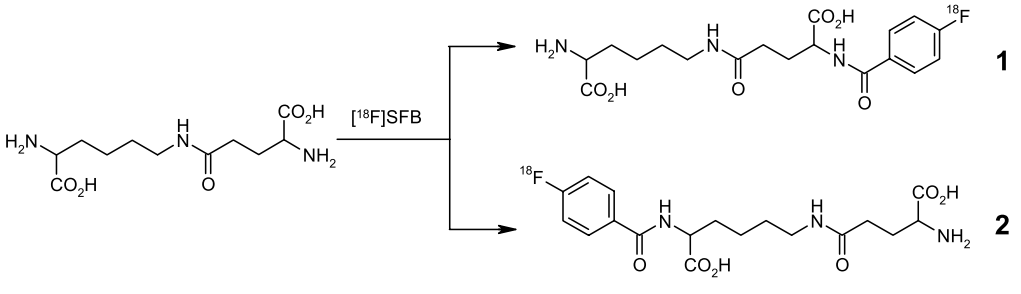

Fig. 1. Radiolabelling of isopeptide N$^{\varepsilon}$-(γ-glutamyl)-L-lysine by conjugation with N-succinimidyl-4-[^{18}F]fluorobenzoate

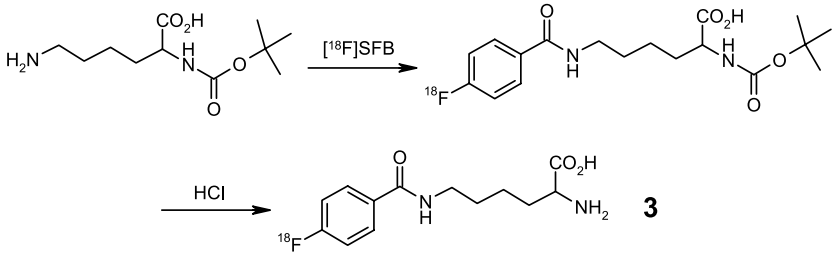

Fig. 2. Radiolabelling of L-lysine by conjugation with N-succinimidyl-4-[^{18}F]fluorobenzoate

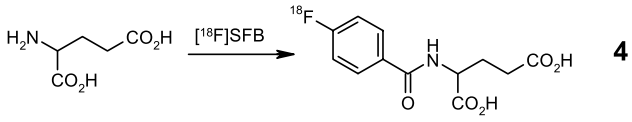

Fig. 3. Radiolabelling of glutamic acid by conjugation with N-succinimidyl-4-[^{18}F]fluorobenzoate

matography with radioactivity detection, the [^{18}F]fluorobenzoylated isopeptides **1** and **2** showed strong differences. While compound **1** remained unchanged over the total incubation period of 90 minutes, the amount of intact compound **2** decreased significantly in all four homogenates examined (Table 1). The fastest degradation of compound **2** occurred in the kidney homogenate, where the substance was diminished within 5 min almost entirely. The half-life of compound **2** in this homogenate was approximately 2.6 min. Furthermore, a rapid transformation of labelling product **2** was also observed in liver homogenate. The half-life was about 8.3 min. A slower conversion of compound **2** was found in pancreas homogenate, where a half-life of approximately 12.5 min was determined. The slowest metabolic conversion of preparation **2** took place in the small intestine homogenate. After 90 min more than 60% of this preparation remained unchanged.

Furthermore, investigations with homogenates which had been heated for 5 minutes at 80°C were performed. Almost no conversion of compound **2** was observed in homogenates from liver, pancreas, and small intestine within 90 min, indicating complete thermal inactivation of enzyme systems. Solely in heated kidney homogenate, a minor degradation of substance **2** took place. Within 90 min approximately one fourth of compound **2** was converted.

The incubation experiments with membranous and cytosolic fraction of kidney homogenate suggest a fast conversion of compound **2** in both fractions. While compound **2** was completely converted within 5 min in the membranous fraction, degradation in cytosolic fraction was slightly slower. After 5 min, 29% of the preparation was still detectable. Differences between membranous and cytosolic fraction can only been seen within the first 30 min. Afterwards, compound **2** was also completely converted in cytosolic fraction.

3.3 Biodistribution studies

Figure 4 shows the biodistribution of the four labelled compounds in Wistar rats 5 min p.i. All four preparations showed similar biodistributions with exception of the kidneys. It is noteworthy that substance **1** exhibits significantly higher accumulation in the kidneys compared to the [^{18}F]fluorobenzoylated isopeptide **2** and [^{18}F]fluorobenzoylated lysine **3**, which resulted in comparable amounts of radioactivity in the kidneys. The lowest radioactivity accumulation in the kidneys was observed for [^{18}F]fluorobenzoylated glutamic acid **4**. Furthermore, the individual radioactive compounds differed remarkably in their elimination. While after application of glutamic acid **4** almost 70% of total radioactivity could be found in

Table 1. Percentage of remaining compounds **1** and **2** after incubation in various tissue homogenates

Time in min	Kidneys		Liver		Pancreas		Small intestine	
	1	**2**	**1**	**2**	**1**	**2**	**1**	**2**
0	100	100	100	100	100	100	100	100
5	100	2	100	61	100	74	100	90
15	100	1	100	28	100	42	100	84
30	100	1	99	11	100	29	100	81
60	98	1	98	3	98	13	98	73
90	98	0	98	1	98	9	98	62

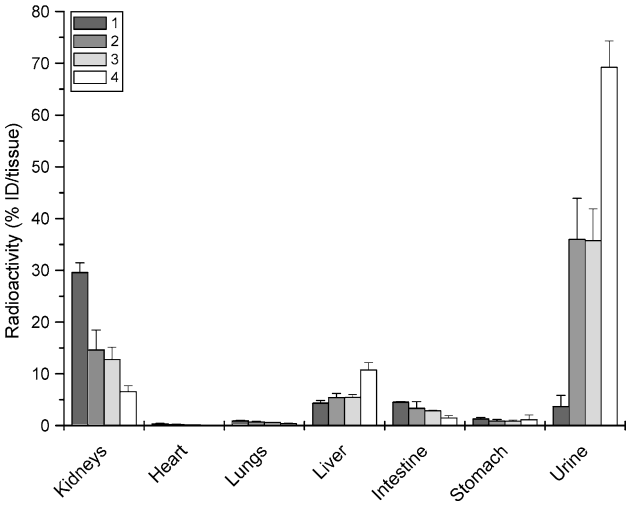

Fig. 4. Biodistribution of ^{18}F-labelled compounds in Wistar rats (mean ± SD) (n = 4) 5 min *p.i.*

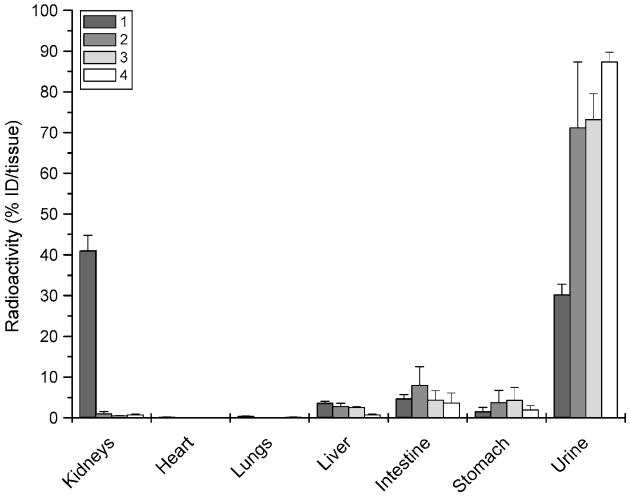

Fig. 5. Biodistribution of ^{18}F-labelled compounds in Wistar rats (mean ± SD) (n = 4) 60 min *p.i.*

urine 5 min p.i., less than 5% of radioactivity were excreted to the urine for [^{18}F]fluorobenzoylated isopeptide **1** at this point of time. Compound **2** and [^{18}F]fluorobenzoylated lysine **3** again exhibited comparable values with a portion of 36% of total radioactivity in urine 5 min p.i. in each case. The distribution of radioactivity in the other organs was similar for all four examined preparations. Figure 5 presents the biodistribution of the four labelled compounds in Wistar rats 60 min p.i.

Again, large differences in the radioactivity enrichment were found for the kidneys after 60 min. While [^{18}F]fluorobenzoylated isopeptide **2**, [^{18}F]fluorobenzoylated lysine **3**, and [^{18}F]fluorobenzoylated glutamic acid **4** were almost completely eliminated from the kidneys 60 min p.i., an increase in radioactivity accumulation in the kidneys from 30% 5 min p.i. to 41% 60 min p.i. concomitant with a substantial lower amount of radioactivity measured for the corresponding urine samples could be observed for [^{18}F]fluorobenzoylated isopeptide **1**. The fastest elimination of radioactivity was noticed for [^{18}F]fluorobenzoylated glutamic acid **4**. One hour after injection of this preparation, more than 87% of radioactivity was excreted into urine.

3.4 PET studies

As shown in Fig. 6, representing coronal PET images of the abdominal region, a fast association of radioactivity in the kidneys for both preparations [^{18}F]fluorobenzoylated isopeptide **1** and **2** was observed, which clearly supports the biodistribution data.

For the [^{18}F]fluorobenzoylated isopeptide **2**, radioactivity is delivered via the renal pelvis to the ureter after a short period of time. 20 min p.i. hardly any radioactivity can be observed for compound **2** in the kidneys. Nearly complete radioactivity can be found in the bladder. In contrast to this, for the [^{18}F]fluorobenzoylated isopeptide **1** an accumulation of radioactivity can be observed in the cortex of the kidney. Even 60 min p.i. the predominant part of radioactivity still remains in the cortex of the kidney.

3.5 In vivo stability

In vivo stability and possible formation of metabolites were investigated using RP-HPLC for all synthesized [^{18}F]fluorobenzoylated compound **1** to **4**. First of all, [^{18}F]fluorobenzoylated glutamic amine acid **4** was examined. Appearance of metabolites could only be observed in urine, where five metabolites were detected, representing less than 4% of the total radioactivity.

For [^{18}F]fluorobenzoylated lysine **3**, metabolites could be found in plasma, urine and kidneys. 60 min p.i., relative amounts of compound **3** were 90% in the plasma sample, 80% in the urine sample and 68% in the kidney sample, respectively. In each of these samples, two metabolites were observed. The more abundant one was identified as 4-[^{18}F]fluorohippuric acid, a conjugation product of 4-[^{18}F]fluorobenzoic acid with glycine. For the [^{18}F]fluorobenzoylated isopeptide **1**, neither in blood nor in kidneys metabolites were detected. In urine, the appearance of numerous metabolites and a remarkable decrease of compound **1** could be noticed. 5 min p.i. only 10% of the original preparation **1** was left in urine, and 60 min p.i. compound **1** was no longer detectable. None of the metabolites was identified. However, the metabolites were not identical to compound **4** or one of its metabolites.

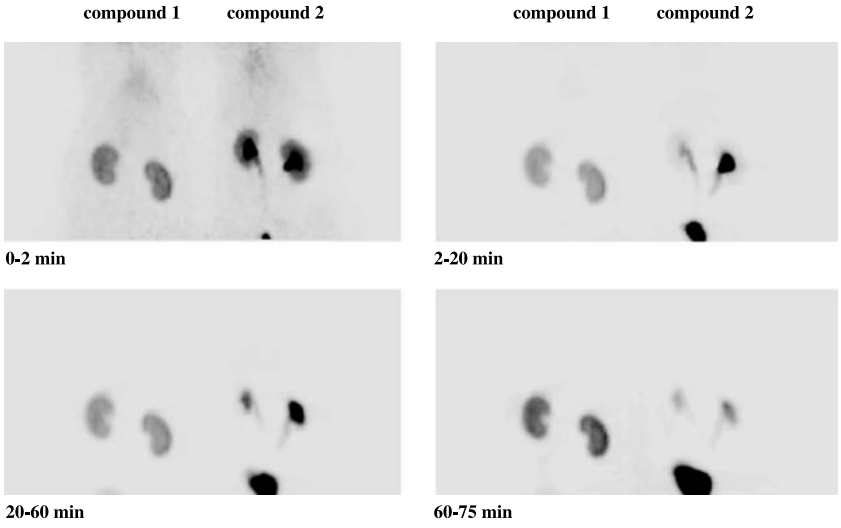

Fig. 6. Representative coronal images of small animal PET studies showing ^{18}F-radioactivity distribution (maximum intensity projection) of abdominal region after application of 15 MBq of compound **1** and **2**, respectively, at various time intervals *p.i.*

For the [^{18}F]fluorobenzoylated isopeptide **2**, metabolites could be found in urine as well as in blood and in kidneys. 60 min p.i., relative amounts of compound **2** in the plasma sample was 64%, in the kidney sample 21% and in urine 18% of total radioactivity. The main metabolite which was found in all samples was identified as [^{18}F]fluorobenzoylated lysine. Furthermore, 4-[^{18}F]fluorobenzoic acid was detected in the kidney samples.

4 Discussion

The bioavailability of isopeptide N$^{\varepsilon}$-(γ-glutamyl)-L-lysine has been reported by several groups based on feeding tests with unlabelled and ^{3}H- or ^{14}C-labelled isopeptide (Waibel and Carpenter, 1972; Raczynski et al., 1975; Yasumoto and Suzuki, 1990; Seguro et al., 1996). However, feeding tests with unlabelled N$^{\varepsilon}$-(γ-glutamyl)-L-lysine exclusively allow conclusions on the biological value. Radiolabellings with ^{3}H ($t_{1/2} = 12.3$ a) and ^{14}C ($t_{1/2} = 5730$ a) have the disadvantage of a very low specific activity. Therefore, certain transporters or other mechanisms can only be examined in the proximity of saturation. As a possible alternative to these experiments, labelling with ^{18}F was performed in the present study. The ^{18}F-labelled compounds used for our biochemical investigations had a specific activity of 10 GBq/μmol after synthesis, isolation, and processing, which is a substantially higher specific activity compared to the substances described in literature. The ^{3}H-labelled substances used by Raczynski and coworkers exhibited a specific activity of 2.2 MBq/μmol (Raczynski et al., 1975). The ^{14}C-labelled compound deployed by Finot and colleagues had a specific activity of 32.6 MBq/μmol (Finot et al., 1978).

The linkage of ^{18}F to the isopeptide N$^{\varepsilon}$-(γ-glutamyl)-L-lysine was achieved via a prosthetic group, namely N-succinimidyl-4-[^{18}F]fluorobenzoate ([^{18}F]SFB). Due to the presence of two primary amino groups in the isopeptide, two labelling products were obtained. It has to be considered that the isopeptide N$^{\varepsilon}$-(γ-glutamyl)-L-lysine is modified by this labelling reaction. Therefore one has to keep in mind that the obtained results of the *in vitro* and *in vivo* examinations are not caused by N$^{\varepsilon}$-(γ-glutamyl)-L-lysine but by [^{18}F]fluorobenzoylated N$^{\varepsilon}$-(γ-glutamyl)-L-lysine. However, these [^{18}F]fluorobenzoylated compounds are likely to act as a model for a tripeptide containing one isopeptide bond. With respect to its steric features, the [^{18}F]fluorobenzoyl group might be compared with a tyrosine or phenylalanine residue.

The results of the *in vitro* examinations of the [^{18}F]fluorobenzoylated N$^{\varepsilon}$-(γ-glutamyl)-L-lysine show a completely different behavior of the labelling products **1** and **2**. Whereas compound **1** remained almost unchanged in all examined tissue homogenates, compound **2** was transformed in every case. The fastest degradation was observed in kidney homogenate, the slowest degradation of labelling product **2** was found in small intestine homogenate. These transformation rates partly correlate with the enzymatic activities of γ-glutamyltranspeptidase and γ-glutamylcyclotransferase in the corresponding tissue reported in the literature (Tate et al., 1985; Szewczuk et al., 1974). Table 2 presents the enzymatic activities of γ-glutamylcyclotransferase in different tissues and the corresponding experimentally determined half-lives for compound **2** in these tissues.

For kidney, pancreas, and intestine, relative activity is in good correlation with half-lives found for compound **2**.

Table 2. Relative enzymatic activity of γ-glutamylcyclotransferase in rat tissues according to Szewczuk and coworkers (Szewczuk et al., 1974) compared with experimentally determined half-lives of compound **2** in rat tissues

Tissue	Relative enzymatic activities	Experimental determined half-lives of compound **2**
Kidney	100	2.6
Liver	9	8.3
Pancreas	24	12.5
Intestine	4	approximately 120

Table 3. Relative enzymatic activity of γ-glutamyltranspeptidase in rat tissues according to Tate and coworkers (Tate et al., 1985)

Tissue	Relative enzymatic activities
Kidney	100
Liver	0.2
Pancreas	20

However, the fast conversion of compound **2** observed in liver homogenate can be explained only partly by γ-glutamylcyclotransferase, as for this organ only small relative activities are reported. Another enzyme which catalyses the cleavage of isopeptide bonds is the γ-glutamyltranspeptidase. Table 3 shows the activities of this enzyme which have been determined in homogenates from different tissues from rats (Tate et al., 1985).

These investigations showed high γ-glutamyltranspeptidase activity in the kidneys and in the pancreas. However, rat liver showed a very low enzymatic activity of γ-glutamyltranspeptidase when compared to the other organs. It therefore appears that the observed transformation rate of compound **2** in liver homogenate can also not be explained by the action of this enzyme. This means that there must be further enzymatic activity in the liver which is able to convert the [^{18}F]fluorobenzoylated isopeptide **2**. Possible candidates in this context might be transglutaminases themselves, because they are known to be predominantly located in the liver. The isopeptidase activity of transglutaminases was reported by Parameswaran and coworkers (Parameswaran et al., 1997). An involvement of other enzymes can also not be ruled out. The finding that degradation of labelling product **2** does not take place in heated homogenates or is at least significantly reduced, thus is supporting the hypothesis that conversion of this compound is actually caused by enzymes.

Furthermore, conversion of compound **2** was investigated in membranous and cytosolic fraction of kidney homogenate. The reason for this experiment was the fact that the enzyme γ-glutamylcyclotransferase is localized in cytosol, while γ-glutamyltranspeptidase can be found membrane-bound. Because of a fast degradation of compound **2** in both membranous and cytosolic fraction, both enzymes might participate in transformation of this [^{18}F]fluorobenzoylated isopeptide. However, an involvement of other enzymes can not be excluded.

Moreover, the *in vivo* metabolism of the two [^{18}F]fluorobenzoylated isopeptides was investigated. Here it could be noticed that compound **1** was transformed in the living organism whereas it remained unchanged in the *in vitro* studies. However, transformation products could only be observed in urine. These transformation products could not be identified, but they are not identical to compound **4** or one of its metabolites. This means that a cleavage of [^{18}F]fluorobenzoylated isopeptide **1** can neither be proven nor excluded. A conversion of this labelling product by γ-glutamylcyclotransferase seems to be improbable because this enzyme catalyzes the reaction shown in Fig. 7. During this reaction the free α-amino group of the γ-glutamyl moiety attacks the carbonyl C-atom of the isopeptide bond, followed by cyclization to oxoproline and hydrolytic liberation of the free amine. It seems unlikely that this cyclization can also take place if the α-amino group of this moiety is substituted by a [^{18}F]fluorobenzoyl group. However, a cleavage of the isopeptide bond of compound **1** by γ-glutamyltranspeptidase is conceivable. The proposed reaction is presented in Fig. 8.

Assuming this reaction for the [^{18}F]fluorobenzoylated isopeptide **1**, the unlabelled lysyl moiety of compound **1** is splitted off and would no longer be traceable by the detection of radioactivity. In the course of this reaction, the γ-glutamyl moiety carrying the ^{18}F-label would be transferred to another amino acid or peptide. The new radioactive amino acids or peptides thereby formed might explain the non-identified substances in the urine. A further splitting of these new [^{18}F]fluorobenzoylated compounds by the γ-glutamylcyclotransferase should not be possible for the reasons mentioned above.

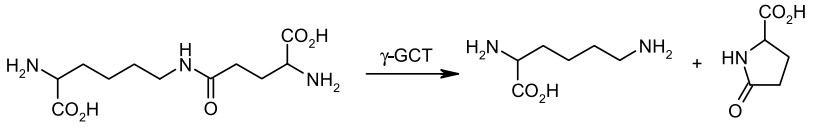

Fig. 7. Conversion of N$^{\varepsilon}$-(γ-glutamyl)-L-lysine by γ-glutamylcyclotransferase (γ-GCT)

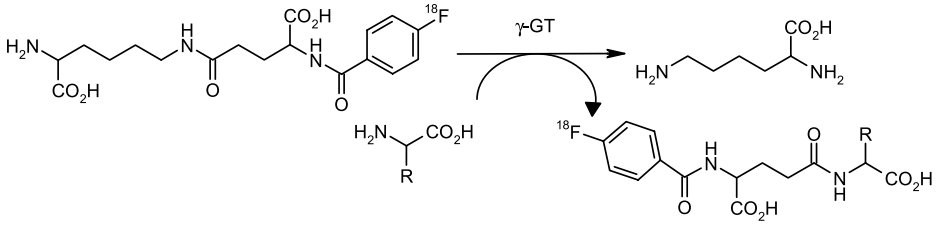

Fig. 8. Proposed conversion of [^{18}F]fluorobenzoylated isopeptide **1** by γ-glutamyltranspeptidase (γ-GT)

In contrast to these findings, a cleavage of the isopeptide bond could be shown for the [^{18}F]fluorobenzoylated isopeptide **2**. Here, [^{18}F]fluorobenzoylated lysine was found in studies *in vitro* as well as in investigations *in vivo*.

In conclusion, the two [^{18}F]fluorobenzoylated derivatives of N$^{\varepsilon}$-(γ-glutamyl)-L-lysine **1** and **2** seem to have different metabolic pathways. This is also supported by the results of the biodistribution and the PET studies, where compound **2** and [^{18}F]fluorobenzoylated lysine **3** showed comparable behavior. Both compounds were taken up fast in the kidneys, but they also were excreted rapidly to the urine. These findings suggest that [^{18}F]fluorobenzoylated isopeptide **2** is cleaved fast, and soon after application of compound **2** the distribution of [^{18}F]fluorobenzoylated lysine could be observed. [^{18}F]fluorobenzoylated glutamic acid also showed a fast kinetics in kidneys. In contrast, the [^{18}F]fluorobenzoylated isopeptide **1** exhibited a quite slow accumulation of radioactivity in the kidneys. Furthermore, excretion of accumulated radioactivity from kidneys was delayed. This means that compound **1** and compound **4** do not show any similarities thereby confirming the results obtained in metabolite analysis.

As mentioned above, it should be noticed that the two labelling products of N$^{\varepsilon}$-(γ-glutamyl)-L-lysine have completely different metabolisms and biodistributions. The present investigations point out a clear influence of the [^{18}F]fluorobenzoyl group on the biochemical behavior of the isopeptides. The results indicate that the *in vivo* behavior of N$^{\varepsilon}$-(γ-glutamyl)-L-lysine particularly depends on the substitution of the two α-amino groups. Therefore, it should be the crucial factor how the isopeptides are really absorbed in the digestive tract – as a dipeptide solely consisting of lysine and glutamine or peptide bound. According to this, different metabolic pathways can be expected. For a better understanding of the metabolism of compound **1**, the identification of its metabolites which were found in urine would be necessary. Because a transfer of the [^{18}F]fluorobenzoylated γ-glutamyl moiety to other amino acids or peptides is expected, the synthesis of various [^{18}F]fluorobenzoylated γ-glutamyl amino acids as reference compounds might be useful. So, it could be ascertained whether compound **1** is still a substrate of the γ-glutamylcyclotransferase. Furthermore, it should be considered that the metabolism of the [^{18}F]fluorobenzoylated compounds was studied after their intravenous application. For that reason, conversions which take place in the intestine or during resorption had not been accounted. Consequently, studies following ingestion of compound **1** and compound **2**, respectively, have to be carried out.

The present study shows a cleavage of the isopeptide bond in case of an unsubstituted α-amino group of the glutamyl moiety. Therefore it can be expected that conventional peptide bonds of a peptide containing an isopeptide bond are cleaved by peptidases soon after absorption, so that N$^{\varepsilon}$-(γ-glutamyl)-L-lysine is liberated and there is no longer a substitution of the α-amino group of the glutamyl moiety. Subsequently the isopeptide bond can be cleaved. Thus, it appears that the use of transglutaminases in food biotechnology and the resulting isopeptides in these finished products do not pose a risk to health of the consumers.

In summary, PET seems to be a very useful tool to study biodistribution and elimination of food compounds, in particular proteins, peptides or amino acids. Furthermore, the detection of radioactive metabolites makes it possible to ascertain the metabolic pathways of these compounds.

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