

# Oxidation of methionine — is it limiting the diagnostic properties of <sup>99m</sup>Tc-labeled Exendin-4, a Glucagon-Like Peptide-1 receptor agonist?

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## Abstract

**BACKGROUND:** Preliminary clinical evaluation of <sup>99m</sup>Tc-EDDA/HYNIC-Met<sup>14</sup>-Exendin-4 showed that the complex offers new diagnostic possibilities for insulinoma and MTC. Exendin-4 contains methionine at position 14 in the amino acid chain, which may be oxidized to methionine sulfoxide and, from the pharmaceutical point of view, the oxidized moiety becomes an undesired impurity in the final radioactive preparation. Therefore, the aim of this study was to investigate the influence of commonly used methods to eliminate the effect of methionine oxidation in peptides, i.e. the replacement of methionine by norleucine (Nle) and the addition of L-methionine, on the in vitro stability and the biodistribution.

**MATERIAL AND METHODS:** <sup>99m</sup>Tc-EDDA/HYNIC-Met<sup>14</sup>-Exendin-4, <sup>99m</sup>Tc-EDDA/HYNIC-Nle<sup>14</sup>-Exendin-4, <sup>99m</sup>Tc-EDDA/HYNIC-Met<sup>14</sup>-Exendin-4 with the addition of L-methionine and an oxidized form of Exendin-4, i.e. <sup>99m</sup>Tc-EDDA/HYNIC-Met<sup>14</sup>(ox)-Exendin-4 were compared in vivo with <sup>68</sup>Ga-NODAGA-Nle<sup>14</sup>-Exendin-4 in normal Wistar rats. The stability and lipophilicity were determined in vitro.

**RESULTS:** Biodistribution studies confirmed the specific uptake of all tested complexes in the GLP-1 positive organs: lungs, pancreas and stomach. The uptake of <sup>99m</sup>Tc-EDDA/HYNIC-Met<sup>14</sup>-Exendin-4 with the addition of L-methionine and for <sup>68</sup>Ga-NODAGA-Nle<sup>14</sup>-Exendin-4 at 1h p.i. was around 2-fold higher than that of <sup>99m</sup>Tc-EDDA/HYNIC-Met<sup>14</sup>-Exendin-4 and <sup>99m</sup>Tc-EDDA/HYNIC-Nle<sup>14</sup>-Exendin-4.

**CONCLUSION:** Although the substitution of methionine by norleucine in the HYNIC-Exendin-4 did not result in improved biodistribution, the use of L-methionine, as the excipient that inhibits the oxidation of methionine in the peptide chain resulted in higher lung/blood and stomach/blood uptake ratios. Our results confirmed that methionine at position 14 of amino acid chain of Exendin-4 plays an important role in the interaction with GLP-1 receptor positive tissue.

**KEY words:** <sup>68</sup>Ga-NODAGA-Nle<sup>14</sup>-Exendin-4, <sup>99m</sup>Tc-EDDA/HYNIC-Met<sup>14</sup>-Exendin-4, <sup>99m</sup>Tc-EDDA/HYNIC-Nle<sup>14</sup>-Exendin-4, L-methionine, GLP-1, methionine sulfoxide

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## Introduction

Insulinomas are rare endocrine tumors. Detection of insulinoma is still very difficult using standard diagnostic techniques. Surgery can be performed only if this small tumor is well localized. The sensitivity of somatostatin receptor scintigraphy (SRS) is only 40–60% due to relatively low incidence of somatostatin subtype receptor sstr2 and sstr5 expression in insulinomas [1]. Importantly, in insulinomas the glucagon like peptide-1 receptors (GLP-1R)

are expressed with high incidence and high density, twice that of sstr2 [2, 3]. The natural agonist of GLP-1R is a 30 amino acid hormone that is rapidly degraded by dipeptidyl peptidase IV [4]. The more stable agonists are Exendin-4 and Exendin-3. Consequently, GLP-1 receptor-avid radioligands have been developed and evaluated [5–8]. In particular, the preclinical animal studies have shown the ability of [Lys<sup>40</sup>(Ahx [6-aminohexanoic acid]-DTPA-<sup>111</sup>In)NH<sub>2</sub>]-Exendin-4 to successfully localize small insulinomas in the Rip1Tag2 mouse tumor model [8] and presented the potential for GLP-1 targeting as therapeutic approach [9]. This new tracer was efficient in localizing the pancreatic tumors and insulinomas, which had previously not been identified using conventional methods [10, 11]. Furthermore, the new Exendin-4 analogs were developed aimed at overcoming the drawbacks of <sup>111</sup>In-imaging. Namely, these were [Lys<sup>40</sup>(Ahx-hydrazinonicotinamide [HYNIC]-

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$^{99m}\text{Tc}$ /ethylenediaminediacetic acid [EDDA] $\text{NH}_2$ -Exendin-4 for SPECT and [ $\text{Lys}^{40}(\text{Ahx-DOTA-}^{68}\text{Ga})\text{NH}_2$ ]-Exendin-4 for PET/CT. The preclinical evaluation of these tracers and [ $\text{Lys}^{40}(\text{Ahx-DTPA-}^{111}\text{In})\text{NH}_2$ ]-Exendin-4 in the Rip1Tag2 mouse model of pancreatic  $\beta$ -cell carcinogenesis revealed that all three of them showed high tumor-to-background ratio, resulting in the visualization of tumors. Very high tumor uptake of [ $\text{Lys}^{40}(\text{Ahx-DOTA-}^{68}\text{Ga})\text{NH}_2$ ]-Exendin-4 at the level  $205 \pm 59$  percentage injected activity per gram tissue at 4h p.i. was observed, while for the other two radiopeptides it was significantly lower. However, a lower tumor and organ uptake of [ $\text{Lys}^{40}(\text{Ahx-HYNIC-}^{99m}\text{Tc/EDDA})\text{NH}_2$ ]-Exendin-4 did not result in the inferior tumor-to-organ ratio or reduced image quality [12]. This prompted us to further develop this tracer into the clinically suitable formulation. Moreover, in our experience, the diagnostic utility of a GLP-1receptor agonist, [ $\text{Lys}^{40}(\text{Ahx-HYNIC-}^{99m}\text{Tc/EDDA})\text{NH}_2$ ]-Exendin-4 obtained from the developed dry kit, has been proven in a small group of patients with insulinoma [13] and MTC [14].

The peptide Exendin-4 contains methionine at position 14 in the amino acid chain, which may be oxidized and, from the pharmaceutical point of view, the oxidized moiety becomes an undesired impurity in the final radioactive preparation. In case of other biomolecules it has been shown that oxidation of methionine to methionine sulfoxide causes their lower bioactivity [15, 16]. Oxidation of methionine present in the amino acid sequence of peptides can have significant influence on the receptor binding affinities. Notably, oxidation of methionine in gastrin analogs dramatically reduces the affinity for CCK-2 receptors [17]. Various approaches were applied to prevent the oxidation of methionine during radiolabeling of peptides, such as addition of antioxidants, including selenomethionine and L-methionine as well as gentisic acid and ascorbic acid [17, 18]. The other option is to replace the methionine with another amino acid, which would not be prone to oxidation. Norleucine is the most commonly used substitute due to its chemical similarity to methionine. The replacement of methionine by norleucine in minigastrin analogs did not significantly affect binding affinities to CCK-2R receptors [19].

Although the development of several radiolabeled Exendin-4 analogs has been reported, the GLP-1 receptor affinities of these analogs, assessed in various cell lines do not differ significantly [8, 12, 19–22]. However, so far, there is no published data on the GLP-1 receptor affinity of the oxidized form of Exendin-4. Therefore, the aim of this work was to investigate the influence of commonly used methods to eliminate the effect of methionine oxidation in peptides, i.e. the replacement of methionine by norleucine (Nle) and the addition of L-methionine to the radiolabeling mixture on the in vitro stability and the biodistribution in normal rats. In this study we characterized and compared the following complexes:  $^{68}\text{Ga}$ -NODAGA-Nle $^{14}$ -Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}$ -Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Nle $^{14}$ -Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}$ -Exendin-4 with the addition of L-methionine and an oxidized form of Exendin-4, i.e.  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}(\text{ox})$ -Exendin-4.

## Material and methods

### Reagents

Chemicals were purchased from Aldrich-Sigma Chemicals Co (Poznan, Poland). Met $^{14}$ , Lys $^{40}(\text{Ahx-HYNIC})$ -Exendin-4 (HYNIC-Met $^{14}$ -Exendin-4), Nle $^{14}$ , Lys $^{40}(\text{Ahx-HYNIC})$ -Exendin-4

(HYNIC-Nle $^{14}$ -Exendin-4), Nle $^{14}$ , Lys $^{40}(\text{Ahx-NODAGA})$ -Exendin-4 (NODAGA-Nle $^{14}$ -Exendin-4) were custom-synthesized by piCHEM (Graz, Austria).  $^{99m}\text{TcO}_4^-$  was obtained from the domestic  $^{99}\text{Mo}/^{99m}\text{Tc}$  generator (Radioisotope Centre POLATOM, Poland).  $^{68}\text{GaCl}_3$  was obtained from  $^{68}\text{Ge}/^{68}\text{Ga}$  generator (IThemba Labs, Republic of South Africa). ITLC SG was purchased from Agilent Technologies (United States).

### Quality control

The radiochemical purity was determined by HPLC and TLC chromatography. HPLC analysis was performed on a Luna 5  $\mu\text{m}$  C18(2) 100A, 4.6 x 250 mm column (Phenomenex) using a Varian system equipped with a dual wave length spectrophotometer set and on-line radioactivity detector connected in series. The chromatographic conditions were as follows: flow rate 1 ml/min, gradient elution: 0 min — 10% B, 10 min — 10% B, 22 min — 50% B, 25 min — 10% B, 30 min — 10% B, mobile phase A: 0.1% TFA in  $\text{H}_2\text{O}$ , B: 0.1% TFA in ACN. The radiochemical purity was expressed as the percentage of the peak corresponding to labeled peptide in the total activity in the radiochromatogram. The injected activity on the HPLC-column was ca. 5 MBq.

Instant TLC (iTLC) was performed using silica gel (iTLC SG-glass microfibre chromatography plates impregnated with silica gel, 2 cm x 10 cm) with 2-Butanone used to determine the amount of free  $^{99m}\text{TcO}_4^-$  ( $R_f = 1$ ), ACN:  $\text{H}_2\text{O}$  (1: 1, v: v) to determine  $^{99m}\text{Tc}$ -colloid ( $R_f = 0$ ) and 0.1 M citrate buffer pH 5.0 to determine the  $^{99m}\text{Tc}$ -Tricine and  $^{99m}\text{Tc}$ -EDDA ( $R_f = 1$ ).

The 0.1 M citrate buffer pH 5.0 was used to determine the free  $^{68}\text{GaCl}_3$  ( $R_f = 0.8$ –1.0) and 5M ammonium acetate : methanol (1: 1, v: v) to determine  $^{68}\text{Ga}$ -colloid ( $R_f = 0$ –0.2). An undiluted sample drop of about 5  $\mu\text{l}$  of radiolabeled Exendin-4 was placed on the start line (1 cm from bottom) and the solvent was allowed to migrate up to 0.5 cm from the top of the plate. The distribution of radioactivity on radiochromatograms was determined using Cyclone<sup>®</sup>Plus (PerkinElmer).

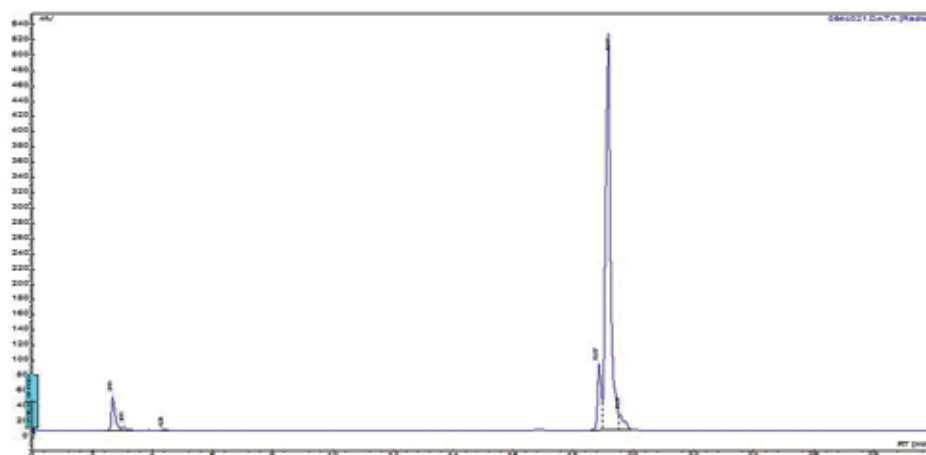
### Radiolabeling

The “wet”  $^{99m}\text{Tc}$ -labeling of HYNIC-Met $^{14}$ -Exendin-4 and HYNIC-Nle $^{14}$ -Exendin-4 was performed to optimize the quantity and concentration of reagents, temperature and reaction time which were then used to develop the dry kit formulation. One-vial dry kit was prepared with the following composition: 30  $\mu\text{g}$  of HYNIC-Met $^{14}$ -Exendin-4 or HYNIC Nle $^{14}$ -Exendin-4, 40  $\mu\text{g}$  of  $\text{SnCl}_2 \cdot x \text{H}_2\text{O}$ , 50 mg of Tricine [N-[Tris(hydroxymethyl)-methyl-glycine] and 5 mg of EDDA (Ethylenediamine-N,N'-diacetic acid). Radiolabeling was performed using the eluate from the  $^{99}\text{Mo}/^{99m}\text{Tc}$  generator eluted within 24h previously.

One-vial kit formulation for the preparation of  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}$ -Exendin-4 with the addition of L-methionine contained: 30  $\mu\text{g}$  of HYNIC-Met $^{14}$ -Exendin-4, 40  $\mu\text{g}$  of  $\text{SnCl}_2 \cdot x \text{H}_2\text{O}$ , 50 mg of Tricine, 30 mg L-methionine and 5 mg of EDDA.

For  $^{99m}\text{Tc}$  labeling, the kit vial was reconstituted with 1.5 ml of eluate (0.7GBq–1.5GBq radioactivity), followed by 10 min incubation at 100°C in a dry block heater. After cooling to room temperature the radiochemical purity of labeled peptide was controlled by TLC and HPLC.

$^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}(\text{ox})$ -Exendin-4 was prepared by oxidation of previously prepared  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}$ -Exendin-4. To



**Figure 1.** HPLC-radiochromatogram  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>-Exendin-4

a 6.58 nM solution of  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>-Exendin-4 100  $\mu\text{l}$  of 30%  $\text{H}_2\text{O}_2$  were added and the mixture was incubated for 15 min at room temperature. Purification was performed using a solid phase extraction column (Oasis<sup>®</sup> HLB 1cc 30mg extraction cartridges). The unbound technetium-99m was eluted with 5 ml of water and  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>(ox)-Exendin-4 was eluted with 1 ml of ethanol. Its identity and radiochemical purity were confirmed by HPLC.

For  $^{68}\text{Ga}$  labeling, 30  $\mu\text{g}$  of NODAGA-Nle<sup>14</sup>-Exendin-4 in 400  $\mu\text{l}$  of 2.5MHEPES[4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid] was incubated with  $^{68}\text{GaCl}_3$  (0.2 GBq–0.5 GBq radioactivity) for 10 min at 100°C. pH of the solution was 3.8–4.0.

#### 2.4. In vitro stability studies

The stability of  $^{68}\text{Ga}$ -NODAGA-Nle<sup>14</sup>-Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>-Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Nle<sup>14</sup>-Exendin-4 and  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>-Exendin-4 with the addition of L-methionine were performed by incubation of 200  $\mu\text{L}$  of radiolabeled peptide in 1000  $\mu\text{L}$  of 0.9% NaCl. The stability was evaluated by reversed phase-HPLC at different time points: 1h, 2h, 4h, and 6h.

#### Octanol/water partition coefficient

The lipophilicity of the  $^{68}\text{Ga}$ -NODAGA-Nle<sup>14</sup>-Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>-Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Nle<sup>14</sup>-Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>-Exendin-4 with the addition of L-methionine and  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>(ox)-Exendin-4 was determined using the octanol/water partition coefficients. To 0.1 ml of radiolabeled Exendin-4 preparation, 0.4 ml of 1-octanol and 0.3 ml of water were added. After being vigorously mixed for 1 min and gently mixed for an additional 10 min, the two phases were separated by centrifugation at 4000 g for 10 min. The aliquots were counted for radioactivity and partition coefficient (P) were calculated as the logarithm of the radioactivity ratio in the octanol and water phases.

#### Biodistribution studies

The animal experiments were approved by The 4th Local Animal Ethics Committee in Warsaw (the authorization number 78/2011 and 18/2015), and were carried out in accordance with the national legislation regarding laboratory animals protection and the principles of good laboratory practice. The animals used in this trial were permitted to food and water *ad libitum*.

For biodistribution experiments 0.15  $\mu\text{g}$  (ca. 32 pM) aliquots of each radiolabeled Exendin-4 preparation were injected, i.v. (tail vein) to normal Wistar rats (male, n = 5 per group) in a volume of 0.2 ml. For receptor blocking, the respective groups were co-injected with 100-fold excess of respective non-labeled compound. During the experiments, the animals were housed in metabolic cages. At 1h post-injection (p.i.) the animals were sacrificed under anesthesia with 3% isoflurane. Samples of blood and selected tissues were collected, weighed and then measured for radioactivity content. The radioactivity in these samples was determined using  $\gamma$ -counter and calculated as a percentage of injected dose (%ID) or as percentage of injected dose per gram (%ID/g).

## Results

#### Radiolabeling and stability study

The labeling yield of  $^{68}\text{Ga}$ -NODAGA-Nle<sup>14</sup>-Exendin-4 was 96% at a specific activity of 63 GBq/ $\mu\text{mol}$ . The labeling yield of  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>-Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Nle<sup>14</sup>-Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>-Exendin-4 with the addition of L-methionine and  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>(ox)-Exendin-4 were 94%, 97% and 96%, 94%, respectively, at a specific activity of ca. 100 GBq/ $\mu\text{mol}$  for each preparation.

Despite the fact that the prepared dry kits were stored at the fridge temperature ( $5^\circ\text{C} \pm 3^\circ\text{C}$ ) after a couple of months the contribution of  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>(ox)-Exendin-4 impurity was observed in HPLC radiochromatograms as an additional peak located in close proximity to the radiolabeled  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>-Exendin-4 (Figure 1). The increase of this impurity was also observed with the time of storage of the radiolabeled preparation. To identify the individual impurities in the HPLC, the components of the labeling mixture, EDDA and tricine were labeled with  $^{99m}\text{Tc}$  and the  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>-Exendin-4 was incubated with 30%  $\text{H}_2\text{O}_2$  to increase oxidation. The retention times of observed impurities were in agreement with a retention time of specified standard as shown in Table 1. In order to reduce the oxidation of methionine in HYNIC-Met<sup>14</sup>-Exendin-4, various quantities of L-methionine were added to the dry kit of HYNIC-Met<sup>14</sup>-Exendin-4. The most pronounced effect of reducing the content of  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>(ox)-Exendin-4 to around 2.5% was obtained after

**Table 1.** Identification of impurities in  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}$ -Exendin-4

No.	Retention time [min]	Substance
1.	2.68	$^{99m}\text{Tc}$ -EDDA
2.	3.05	$^{99m}\text{Tc}$ -Tricine
3.	4.35	$^{99m}\text{TcO}_4^-$
4.	18.87	$^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}$ (ox)-Exendin-4
5.	19.17	$^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}$ -Exendin-4
6.	19.57	Unidentified

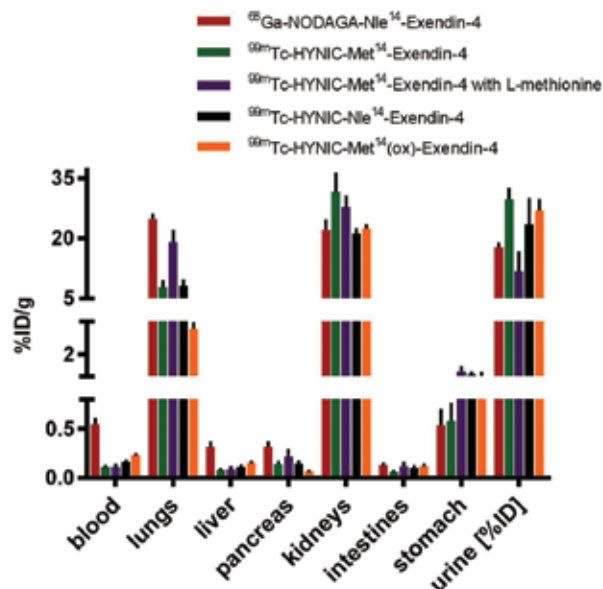
addition of 30 mg of L-methionine and was not further improved with higher mass of L-methionine, therefore 30 mg was used in the biological assays. The effect of L-methionine on radiochemical purity is presented in Table 2.

### Lipophilicity

The Log(P) values for  $^{68}\text{Ga}$ -NODAGA-Nle $^{14}$ -Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}$ -Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Nle $^{14}$ -Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}$ -Exendin-4 with L-methionine and  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}$ (ox)-Exendin-4 were:  $-1.92 \pm 0.01$ ,  $-2.33 \pm 0.004$ ,  $-1.95 \pm 0.007$ ,  $-2.17 \pm 0.005$  and  $-2.79 \pm 0.05$ , respectively. These results indicate that all compounds are hydrophilic, although the oxidized Exendin-4 was the most hydrophilic.

### Biodistribution studies

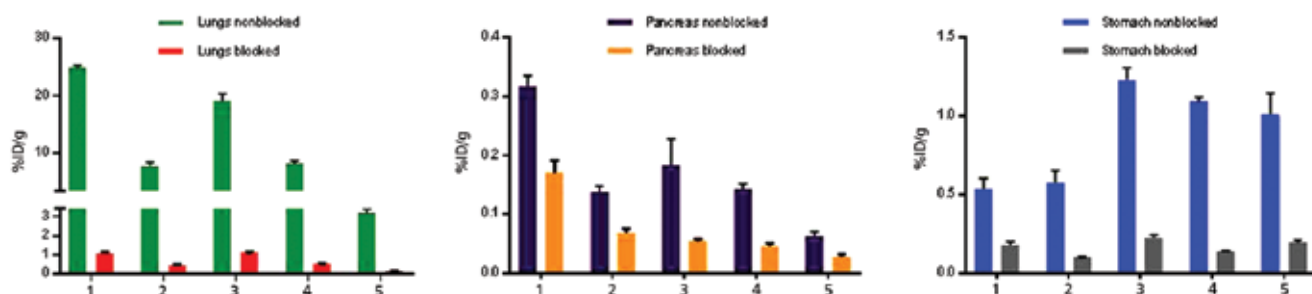
The biodistribution of  $^{68}\text{Ga}$ -NODAGA-Nle $^{14}$ -Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}$ -Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Nle $^{14}$ -Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}$ -Exendin-4 with L-methionine and  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}$ (ox)-Exendin-4 in Wistar rats at 1h p.i. is summarized in Figure 2. All tested radiopeptides showed high uptake of radioactivity in the GLP-1 receptor positive organs, such as the lungs, pancreas and stomach. This uptake was shown to be receptor specific, given the significant reduction of the uptake in the group of animals receiving an excess of respective

**Figure 2.** Biodistribution at 1h p.i. in normal Wistar rats (n = 5) of  $^{68}\text{Ga}$ -NODAGA-Nle $^{14}$ -Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}$ -Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Nle $^{14}$ -Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}$ -Exendin-4 with L-methionine and  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}$ (ox)-Exendin-4 (mean values of %ID/g  $\pm$  SD; n = 5 for each compound)

“cold” Exendin-4 compound (15  $\mu\text{g}$ ) (Figure 3). Out of the five compared preparations, the  $^{68}\text{Ga}$ -NODAGA-Nle $^{14}$ -Exendin-4 and  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}$ -Exendin-4 with L-methionine showed the highest and comparable uptake in the GLP-1 receptor rich organs (lungs:  $24.81 \pm 0.95\%$  ID/g and  $19.08 \pm 2.75\%$  ID/g, pancreas:  $0.32 \pm 0.04\%$  ID/g and  $0.22 \pm 0.06\%$  ID/g, stomach:  $0.53 \pm 0.16\%$  ID/g and  $1.23 \pm 0.17\%$  ID/g, respectively). Two other analogs,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}$ -Exendin-4 and  $^{99m}\text{Tc}$ -EDDA/HYNIC-Nle $^{14}$ -Exendin-4 showed similar uptake in GLP-1 positive organs; however, it was more than twice lower than for the first

**Table 2.** The influence of L-methionine on the radiochemical purity of  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}$ -Exendin-4

L-Methionine [mg]	0	5	10	20	30	40
Radiochemical purity:						
$^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}$ -Exendin-4 (%)	$90.26 \pm 0.2$	$91.63 \pm 0.5$	$92.18 \pm 0.9$	$94.40 \pm 0.7$	$96.17 \pm 0.5$	$96.46 \pm 1.1$
$^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}$ (ox)-Exendin-4 (%)	$9.07 \pm 0.3$	$7.54 \pm 0.6$	$5.69 \pm 0.7$	$3.72 \pm 0.4$	$2.49 \pm 0.6$	$2.54 \pm 0.9$

**Figure 3.** Biodistribution at 1h p.i. in normal Wistar rats (n = 5) of  $^{68}\text{Ga}$ -NODAGA-Nle $^{14}$ -Exendin-4 (1),  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}$ -Exendin-4 (2),  $^{99m}\text{Tc}$ -EDDA/HYNIC-Nle $^{14}$ -Exendin-4 (3),  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}$ -Exendin-4 with L-methionine (4) and  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met $^{14}$ (ox)-Exendin-4 (5) alone and blocked accumulation in the positive organs by co-injection of 15  $\mu\text{g}$  unlabeled Exendin-4

two (lungs:  $7.75 \pm 1.41\%$  ID/g and  $8.11 \pm 1.24\%$  ID/g, pancreas:  $0.14 \pm 0.02\%$  ID/g and  $0.14 \pm 0.02\%$  ID/g, stomach:  $0.58 \pm 0.17\%$  ID/g and  $1.09 \pm 0.06\%$  ID/g, respectively). It should be noticed that for the  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>(ox)-Exendin-4, the uptake in the lungs was  $3.17 \pm 0.51\%$  ID/g, in pancreas:  $0.06 \pm 0.01\%$  ID/g, which is four times lower than for the first two and twice lower than for the other two preparations. The renal uptake of all tested radiolabeled peptides was at the level of more than 20%, similar for all preparations. It was not blocked by an excess of "cold" Exendin-4 (data not shown), indicating that the renal uptake is not GLP-1 mediated.

## Discussion

$^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>-Exendin-4, obtained from the dry kit formulation, has been proven useful in SPECT imaging of insulinomas and MTC [13, 14]. Therefore, the development of the kit formulation for EDDA/HYNIC-Met<sup>14</sup>-Exendin-4 for labeling with technetium-99m was an important achievement in our studies. However, we observed that methionine in Exendin-4 when oxidized during storage, what resulted in decreasing the radiochemical purity of the preparation after radiolabeling with  $^{99m}\text{Tc}$ . Typically, the recommended limit of RCP for the  $^{99m}\text{Tc}$ -labeled preparations, such as  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>-Exendin-4 or  $^{99m}\text{Tc}$ -EDDA/HYNIC-Nle<sup>14</sup>-Exendin-4, is > 90% and the specified impurities are related to the radiochemical forms of  $^{99m}\text{Tc}$  (either as free pertechnetate or reduced-hydrolyzed forms). The oxidized form of the peptide constitutes another impurity and worsens the overall radiochemical purity of the preparation. It is known from others experience that the application of an antioxidant such as selenomethionine, gentisic acid, ascorbic acid or L-methionine affects the inhibition of methionine oxidation in peptides [17, 23, 24]. Using this approach we were also able to improve the stability of HYNIC-Met<sup>14</sup>-Exendin-4 during storage of the dry kit by addition of L-methionine as an antioxidant to the dry kit formulation and also prevent the formation of the oxidized form in the final radioactive preparation.

Although the development of several radiolabeled Exendin-4 analogs has been reported, the receptor affinities of these analogs to certain cell lines do not differ significantly. However, so far, there is no published data on the GLP-1 receptor affinity of the oxidized form of Exendin-4. The introduction of chelator in various lysine positions gave compounds with comparable biological activity with the in vitro binding affinities to GLP-1R in the range of 29 to 54 nM; however, only the lysines at positions 12 and 40 were suggested as preferential modification site [18]. In another study, both lysine residues appeared to be important for the affinity to the GLP-1 receptor [25]. Other modifications like e.g. blocking the SH group of cysteine at position 40 by the introduction of MAL linkage did not change biological activity, but reduced accumulation in the kidneys [26].

On the other hand, it is well known, that oxidation of methionine causes a significant change in its biophysical properties and often leads to an alteration of protein functions. Methionine has a long and non-polar side chain, which becomes polar upon oxidation. The hydrophobicity index of MetO has been estimated to be similar to that of Asn (asparagine), as it has been found for gastrin analogs. Therefore, the oxidation of Met can be considered as a substitution of a hydrophobic for a hydrophilic amino acid and is expected to

have pronounced structural and functional consequences. Herein reported results of partition coefficient confirmed that the preparation of Exendin-4 with oxidized Met<sup>14</sup> was the most hydrophilic of all tested preparations, whereas the  $^{68}\text{Ga}$ -NODAGA-Nle<sup>14</sup>-Exendin-4 was the most lipophilic, probably due to the highly, in relation to methionine, hydrophobicity of norleucine.

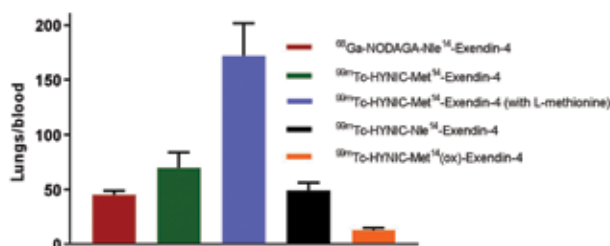
These differences had impact on the biodistribution of evaluated tracers in our studies. It should be noted that in all cases the GLP-1 receptor specific uptake was observed in the organs naturally overexpressing these receptors, such as the lungs, pancreas and stomach. This uptake could be blocked by the coinjection of the excess of "cold" peptide. However, as presented in Figure 3, this uptake was the lowest for the oxidized form of Exendin-4, suggesting that the receptor affinity is enhanced by methionine oxidation. In our study  $^{68}\text{Ga}$ -NODAGA-Nle<sup>14</sup>-Exendin-4 served as an internal standard for comparison of specific uptake to the GLP-1R expressing organs since it was well characterized previously both in terms of receptor binding affinity and biodistribution and tumor visualization in animals [22]. High receptor affinity was reported for [<sup>68</sup>Ga-NODAGA-Nle<sup>14</sup>-Exendin-4], the IC<sub>50</sub> value was  $2.17 \pm 0.42$  nM while for native GLP-1 it was  $1.0 \pm 0.2$  nM. The same authors reported the log (P) value for  $^{68}\text{Ga}$ -NODAGA-Nle<sup>14</sup>-Exendin-4 of  $-2.38 \pm 0.037$ , confirming its hydrophilic character.

The uptake in GLP-1 negative tissues such as liver was low in all  $^{99m}\text{Tc}$ -labeled Exendin-4 formulations. Relatively high uptake of  $^{68}\text{Ga}$ -NODAGA-Nle<sup>14</sup>-Exendin-4 in the liver can be explained by  $^{68}\text{Ga}$  release from the chelator due to the transchelation with blood cells [27]. In case of  $^{68}\text{Ga}$ -labeled radiopeptide, the radioactive metabolites formed and free gallium can potentially negatively affect the imaging quality and increase the dose to non-target tissue. The low hepatic background level for  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>-Exendin-4 with L-methionine is noteworthy since liver is a major site for potential metastasis from insulinoma.

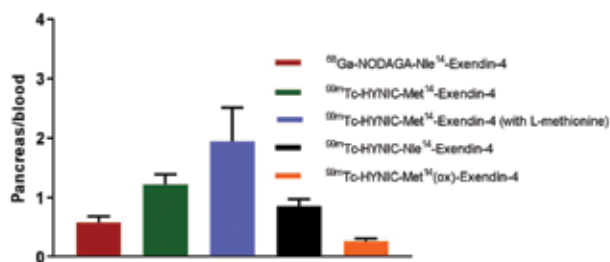
$^{68}\text{Ga}$ - and  $^{99m}\text{Tc}$ -labeled Exendin-4 analogs are characterized by a very high kidney uptake [28]. In our study, the coinjection of an excess of "cold" Exendin-4 did not change renal uptake of  $^{68}\text{Ga}$  and  $^{99m}\text{Tc}$  Exendin-4 analogs, indicating that it was not GLP-1R specific in this tissue, since other mechanisms are involved [29–32]. Tracers for GLP-1R imaging are excreted primarily through the kidneys and can be partially absorbed in the proximal tubule cells. The radiation dose to the kidneys can cause renal toxicity, hence limiting their use for peptide receptor radionuclide therapy.

The rapid clearance from the blood observed for all tested preparations resulted in high lungs/blood uptake ratio. The highest ratio was  $172.7 \pm 29.7$  for  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>-Exendin-4 with L-methionine (Figure 4). The high value of lungs/blood uptake ratio points to the great potential diagnostic for  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>-Exendin-4 with L-methionine. The value of pancreas/blood ( $1.9 \pm 0.5$ ) and stomach/blood ( $11.0 \pm 0.9$ ) uptake ratio is much lower; however, it was again the highest for  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>-Exendin-4 with L-methionine (Figures 5, 6).

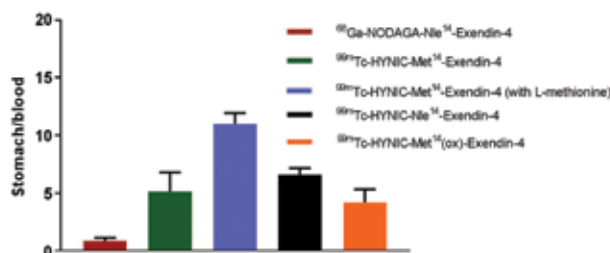
The observed oxidation of methionine in  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>-Exendin-4 reduced the accumulation of the radioactivity in the GLP-1 positive organs compared to the same peptide with addition of L-methionine. Interestingly, the replacement of methionine in the peptide chain of Exendin-4 by norleucine did not increase the accumulation of  $^{99m}\text{Tc}$ -EDDA/HYNIC-Nle<sup>14</sup>-Exendin-4 in the GLP-1 rich organs, although a slight increase in receptor affinity



**Figure 4.** Lungs/blood uptake ratio for  $^{68}\text{Ga}$ -NODAGA-Nle<sup>14</sup>-Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>-Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Nle<sup>14</sup>-Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>-Exendin-4 with L-methionine and  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>(ox)-Exendin-4 at 1h p.i.



**Figure 5.** Pancreas/blood uptake ratio for  $^{68}\text{Ga}$ -NODAGA-Nle<sup>14</sup>-Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>-Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Nle<sup>14</sup>-Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>-Exendin-4 with L-methionine and  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>(ox)-Exendin-4 at 1h p.i.



**Figure 6.** Stomach/blood uptake ratio for  $^{68}\text{Ga}$ -NODAGA-Nle<sup>14</sup>-Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>-Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Nle<sup>14</sup>-Exendin-4,  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>-Exendin-4 with L-methionine and  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>(ox)-Exendin-4 at 1h p.i.

compared to the  $^{111}\text{In}$ -DTPA-Met<sup>14</sup>-Exendin-4 was reported for the  $^{68}\text{Ga}$ -NODAGA-Nle<sup>14</sup>-Exendin-4.

## Conclusion

In this work we have shown that addition of methionine to prevent oxidation of  $^{99m}\text{Tc}$ -EDDA/HYNIC-Met<sup>14</sup>-Exendin-4 allows improving the biodistribution profile of this potential radiopharmaceutical compared with the non-protected formulation. Another approach to obtain similar effect, utilizing norleucine as a replacement for methionine resulted in  $^{99m}\text{Tc}$ -EDDA/HYNIC-Nle<sup>14</sup>-Exendin-4; however, this peptide did not appear to present more favorable biodistribution. This might confirm that presence of methionine in the phar-

macophore of Exendin-4 is crucial for its affinity to GLP-1 receptors. However, this conclusion requires further verification.

## Conflict of interest

The authors declare that they have no conflict of interest.

## Acknowledgments

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