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Neurotensin(8–13) analogue: radiolabeling and biological evaluation using different chelators

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Abstract

Introduction: Several strategies on the development of radiopharmaceuticals have been employed. Bifunctional chelators seem to be a promising approach since high radiochemical yields as well as good in vitro and in vivo stability have been achieved. To date, neurotensin analogs have been radiolabeled using the ^{99m}Tc-carbonyl approach and none was described employing the bifunctional chelating agent technique.
Aim: The purpose of this study was to evaluate the radiochemical and biological behaviour of NT(8–13) analogue radiolabeled with ^{99m}Tc, using HYNIC and NHS-S-acetyl-MAG_3 as chelator agents.
Methods: Radiolabeling, in vitro stability toward cysteine and glutathione, partition coefficient and plasma protein binding were assessed for both radioconjugates. Biodistribution in healthy Swiss mice were carried out in order to evaluate the biological behaviour of the radiocomplexes.
Results: Radiochemical yields were higher than 97% and no apparent instability toward transchelant agents was observed for both radioconjugates. A higher lipophilic character was observed for the radioconjugate labeled via MAG_3. The chelators seem to have no effect on the percentage of the radioconjugate bound to plasma proteins. A similar biological pattern was observed for both radioconjugates. Total blood, bone and muscle values revealed a slightly slower clearance for the radiocomplex labeled via MAG_3. Moreover, a remarkable liver and intestinal uptake was observed for the radiocomplex labeled via MAG_3 even at the later time points studied.
Conclusion: The high radiochemical yields achieved and the similar in vivo pattern found for both radioconjugates make them potential candidates for imaging tumors using nuclear medicine techniques.
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Keywords: Bifunctional chelating agents; Neurotensin analog; ^{99m}Tc

1. Introduction

Diagnostic radiopharmaceuticals are mostly formed by metal complexes with a chelator-biomolecule conjugate. The bifunctional chelating agent (BFCA) is covalently attached to the targeting molecule either directly or through a pharmacokinetic modifying linker, and the radiometal is strongly coordinated by the BFCA. Its choice is largely dependent on the nature and the oxidation state of the radiometal [1]. 6-Hidrazinonicotinamide (HYNIC) and *N*-hydroxysuccinimide mercaptoacetyltriglycine (NHS-MAG_3) have been extensively used as BFCAs for radiorhenium and technetium-99m (^{99m}Tc) labeling of biomolecules such as peptides [2], antibodies [3] and oligonucleotides [4].

Abrams et al. [5] first reported the use of HYNIC as BFCA for ^{99m}Tc labeling. It is known that HYNIC can only occupy one or two coordination sites in the ^{99m}Tc core [6]. In this sense, the use of coligands such as tricine [7], ethylenediamine diacetic acid (EDDA) [8] and bidentate nitrogen-sulfur donors [6], for example, is necessary. Although high radiochemical yields were achieved using this approach, a poor stability in solution of ^{99m}Tc-conjugates was found [9]. On the other hand, ^{99m}Tc-MAG_3 was firstly introduced as a substitute for I-131-*o*-iodohippuric acid as a renal function and imaging agent [10]. Amidothiol chelators are normally prepared with a benzoyl group protecting the thiol.

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Nevertheless, high temperatures or basic environments are needed for deprotection and immediate chelation with the radionuclide, making it inappropriate for the radiolabeling of molecules sensitive at these conditions [11]. In order to circumvent this problem, Winnard et al. [12] developed a synthesis of an *N*-hydroxysuccinimidyl ester of *S*-acetyl-MAG_3 in which the thiol is protected with an acetyl group where the deprotection step takes place at room temperature and neutral pH.

Small neuropeptides are currently the state-of-the-art tool in the development of new site-directed radiopharmaceuticals. Lower molecular weight, faster washout from the bloodstream and better tumor-to-background ratios at early times than high-molecular-weight compounds like monoclonal antibodies are intrinsic characteristics that make peptides the subjects of choice in diagnostic imaging. In addition, peptides are important regulators of growth and cellular functions not only in normal tissues but also in tumors [1,13,14]. However, the major drawback faced by using peptides is the fast metabolization in plasma by endogenous peptidases under physiological conditions [15].

Neurotensin (NT) is a tridecapeptide first isolated from bovine hypothalamus by Carraway and Leeman [16] in 1973. It is known to play a crucial role as a neurotransmitter in the central nervous system (CNS) and as a neuromodulator in the periphery. These actions are mediated by interactions with specific receptors found at the cell membrane of target cells [17]. Since its discovery, several efforts have been performed for investigating structure/activity correlations, indicating that the C-terminal hexapeptide sequence NT(8–13) is essential and could not be changed and/or removed without a complete loss of biological activity [18]. As mentioned above, the in vivo stability can be limited by the action of peptidases. Garcia-Garayoa et al. [19] already designed a series of analogues in order to enhance the biological properties of NT by proposing a double stabilization of the cleavage sites. In attempt to better evaluate the biological behaviour in tumor models of these analogs, they were labeled using the ^{99m}Tc-carbonyl core.

Although the Tc-carbonyl approach usually forms either well-chemically characterized and remarkable stable complexes in aqueous solution [20], the advantages in the use of the BFCA technique over the Tc-carbonyl approach could be attractive. For example, although Tc-carbonyl commercial kits are readily available currently, the two steps involved in the preparation of the final product and the additional purification step prior to further evaluations seem to be unsuitable to the prerequisites argued by the daily routine of clinical radiopharmacies.

In this sense, since the radiolabeling of NT(8–13) analogues with ^{99m}Tc was only reported using the tricarbonyl approach, the purpose of this study was to evaluate the radiochemical and biological behaviour of NT(8–13) analogue [Arg-N(CH_3)-Arg-Pro-Tle-Leu] radiolabeled with ^{99m}Tc, employing the bifunctional chelating agents HYNIC and NHS-S-acetyl-MAG_3. In addition, the establishment of the in vivo pharmacokinetic profile of both radiopharmaceuticals will serve as a pointer for future studies in tumor models.

2. Material and methods

2.1. Chemicals and equipment

Unless otherwise specified all chemicals were of reagent grade and used without further purification (Sigma-Aldrich or Merck). HYNIC-βAla-NT(8–13) [HYNIC-NT(8–13)] and NHS-Sacetyl-MAG_3-βAla-NT(8–13) [MAG_3-NT(8–13)] analogues were purchased from piChem (Vienna, Austria) with purity greater than 99%. Electron spray-mass spectrometry (ES-MS) was carried out according to the expected formulae [mass/charge (*m*/*z*)=1038.3 and 1190.2 for HYNIC and MAG_3 conjugates, respectively]. $Na^{99m}TcO_4$ was eluted in saline solution from an alumina-based ^{99m}Mo/^{99m}Tc generator supplied by the Center of Radiopharmacy (IPEN/CNEN, Sao Paulo, SP, Brazil). Radiochemical control was performed by instant thin-layer chromatography on silica gel strips (ITLC-SG, Gelman Science, Inc., Ann Arbor, MI, USA) and by reverse phase-high performance liquid chromatography (RP-HPLC, Shimadzu, Kyoto, Japan). This analysis was performed using a C-18 column (5.0 μm, 100 Å, 4.6×250 mm, Waters, Milford, MA, USA). All solvents used in chromatographic analyses were HPLC grade and were previously filtered through 0.22-μm membrane filters (Millipore, Milford, MA, USA). Swiss mice for biodistribution studies were provided by the Animal Facility of IPEN-CNEN (Sao Paulo, Brazil).

2.2. Radiolabeling

2.2.1. Radiolabeling of HYNIC-βAla-NT(8–13)

The labeling procedure was done as previously described by our group [21]. Briefly, 0.96 mM (10 μl) of HYNIC-NT (8–13) peptide was added to a sealed reaction vial containing 20 mg tricine and 5 mg of EDDA in 500 μl of 0.1 M of nitrogenated phosphate buffer solution. Then, 5 μl of 8.9 mM $SnCl_2{\cdot}2H_2O$ solution in 0.1N HCl (nitrogen purged) followed by 500 μl of $Na^{99m}TcO_4$ (74- 1850 MBq) was added. The labeled mixture was heated at 100°C for 20 min and cooled to room temperature. The pH of the reaction mixture was 7. Radiochemical yields were determined by ITLC and confirmed by RP-HPLC. For ITLC tests, samples were applied on ITLC-SG strips, developed up to 10 cm from the origin with methylethylketone (MEK) for detection of $^{99m}TcO_4^-$ (R_f=1) and 50% acetonitrile (ACN) for detection of TcO_2 (R_f=0). RP-HPLC solvents consisted of 0.1% trifluoroacetic acid (TFA) in water (Solvent A) and 0.1% TFA in acetonitrile (Solvent B). The HPLC gradient system started with a solvent composition of 95% A/5% B and followed a linear gradient of 30% A/70% B from 0 to 25 min and 30% A/70% B to 5% A/95% B from 25 to 30 min at a flow rate of 1 ml/min.

2.2.2. Radiolabeling of NHS-S-acetyl-MAG$_3$-βAla-NT(8–13)

Radiolabeling of MAG$_3$-NT(8–13) was performed according to the Wang et al. [22] protocol. 0.84 mM of MAG$_3$-peptide (10 μl) was mixed with a combined solution of 45 μl (0.25 M) ammonium acetate, 15 μl of disodium tartrate dihydrate (50 μg/μl in 0.50 M sodium bicarbonate, 0.25 M ammonium acetate and 0.18 M ammonium hydroxide, pH 8.5–9.0) followed by 5 μl of the freshly prepared 4 mg/ml stannous chloride solution in ascorbate-HCl solution. To this, no more than 400 μl of $^{99m}TcO_4^-$ (74–1850 MBq) was added. The labeled mixture was then heated at 100°C for 15–20 min. The final pH was about 7.0. Radiochemical purity was checked by TLC and RP-HPLC as described above.

2.3. In vitro stability assay and transchelation toward cysteine and glutathione

Radiochemical stability was observed in 0 (control), 6- and 24-h time points at room temperature for both ^{99m}Tc-conjugates. For transchelation studies, peptide concentrations were adjusted to 6.4 and 5.3 μM for HYNIC-NT (8–13) and MAG$_3$-NT(8–13), respectively. Equal volumes (100 μl) of cysteine or glutathione solutions were mixed with the radiotracer and incubated at 37°C for 4 h. The challenge agent/peptide molar ratio varied from 1000:1 to 1:1. As control, a vial containing water plus radiotracer was also incubated at the same conditions. Experiments were performed three times and radiochemical purity was checked by TLC. Radiochemical impurities were detected using a solvent system composed of MEK to detect ^{99m}Tc-cysteine/glutathione (R_f=1), $^{99m}TcO_4^-$ (R_f=1) and 50% ACN to detect $^{99m}TcO_2$ (R_f=0). ^{99m}Tc-cysteine/glutathione levels were estimated by the difference between incubated solutions and control.

2.4. Partition coefficient

A total of 200 μl of ^{99m}Tc-labeled peptides (>99%) was added in a flask containing 3 ml each of *n*-octanol and water. The flask was vortexed for approximately 1 min at room temperature. After separation of the phases by gravity, aliquots of 200 μl of each phase (in triplicate) were collected, transferred to separate tubes and taken for γ-radioactivity measurement. Partition coefficient was determined by the function:

$$\text{Partition coeficient} = \text{Log}_{10}\left(\text{counts in octanol layer} / \text{counts in aqueous layer}\right) \quad (1)$$

2.5. Plasma protein binding

The percentage of the radioconjugates bound to plasma proteins was evaluated by the precipitation method. Blood samples were obtained from healthy Swiss mice while under ether anesthesia 5 min postinjection (pi) of ^{99m}Tc-labeled conjugates. Aliquots were collected from the heart in heparinized polypropylene tubes and centrifuged (1877×*g*) for 15 min at room temperature. To 200 μl of plasma, 1 ml of trifluoroacetic acid (TCA) was added, incubated for 30 min in ice bath and centrifuged (2815×*g*) for 30 min at 4°C. Then the supernatant was discarded and the procedure was repeated twice. After the final centrifugation, the pellet was taken for γ-counting along with the standard, which corresponds to the intact plasma (200 μl). The percentage of plasma protein binding of each ^{99m}Tc-labeled conjugate was determined by Eq. (2). The experiments were conducted in triplicate.

$$\%\text{Plasma protein binding} = \left(\text{counts in pellet} / \text{counts in standard}\right) \times 100 \quad (2)$$

2.6. Biodistribution studies

All animal studies were performed at the Center of Radiopharmacy, IPEN/CNEN, and the protocol was approved by the local Animal Welfare Committee.

^{99m}Tc-labeled preparations (111–185 MBq/ml, 0.1 ml) were administered into the tail vein in healthy female Swiss mice (body mass 20–25 g). Fractions of blood were collected from the heart (under ether anesthesia), and then animals were sacrificed by cervical dislocation (six per group) at 5, 30, 60, 90, 120, 240, 360, 1440 min pi. Organs and tissues of interest were excised, weighed and the radioactivity was determined by γ-counting. Results were expressed as percentage of injected dose per gram (%ID/g) and per organ (% ID/organ) of tissue. Total blood, muscle and bone uptake was calculated assuming 7%, 40% and 10% of the total body weight, respectively.

2.7. Statistical analysis

A multilevel factorial design was used as statistical tool for determining the influence of three variables under analysis: BFCAs (HYNIC and MAG$_3$), excised organs and time points from biodistribution studies, as well as their interactions on drug uptake. Six animals were replicated in 16 assessed combinations.

Differences among the considered levels for each variable were detected using the multiple range test of Tukey. The signification level (α) for all statistical tests was 0.05. Experimental planning and statistical analysis were performed with Statgraphics Plus (Statistical Graphics Co., Rockville, MD, USA).

3. Results

3.1. Radiolabeling

Radiochemical yields based on TLC findings were greater than 98% for both radiocomplexes (Table 1). The highest specific activity reached was 191.5 and 219.5 MBq/nmol for the radioconjugates labeled via HYNIC and MAG$_3$,

Table 1
Radiochemical yield (%) of the radiolabeled conjugates based on TLC findings

Product	Time (h)		
	0 (control)	6	24
HYNIC-NT(8–13)	99.45±0.32	98.77±0.15	98.27±0.57
MAG_3-NT(8–13)	99.23±0.62	99.01±0.73	98.47±0.85

Results are expressed as mean±S.D., n=3.

respectively. These findings were confirmed by RP-HPLC. The radiochromatograms showed a single peak, with a retention time of 12.5, 14.7 and 5.3 min for HYNIC-NT (8–13), MAG_3-NT(8–13) and $^{99m}TcO_4^-$ respectively. Less than 1% of total radiochemical byproduct formation, comprising the presence of $^{99m}TcO_4^-$, was also detected (Fig. 1).

3.2. In vitro stability and transchelation toward cysteine and glutathione

Radiochemical stability was monitored during 24 h. Both radioconjugates showed a good stability and less than 2% of radiochemical impurities were observed even for the later time point studied (Table 1).

Challenge experiments with up to 1000-fold molar excess of cysteine or glutathione showed no degree of transchelation for both radiocomplexes during 4 h at 37°C. Only less than 2% of radiochemical impurities ($^{99m}TcO_4^-$ and ^{99m}Tc-cysteine) were detected by challenging ^{99m}Tc-MAG_3-NT(8–13) with cysteine at the higher molar ratio studied (1000:1).

3.3. Partition coefficient

The lipo-hydrophilic character of the radioconjugates was evaluated based on the octanol/water partition coefficients. The log P values were −3.78±0.17 and −1.79±0.04 (mean±S.D.) for HYNIC-NT(8–13) and MAG_3-NT(8–13), respectively, arguing for a higher hydrophilic character for the former conjugate.

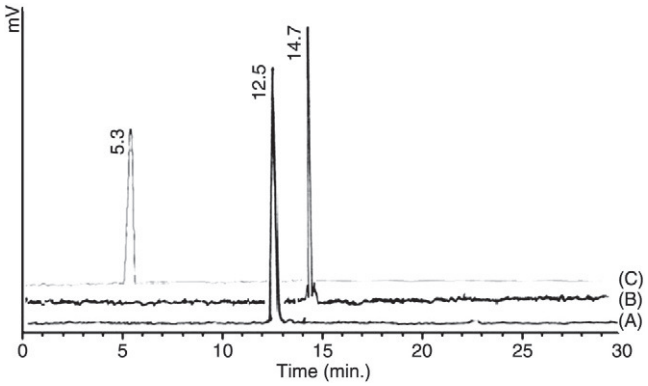


Fig. 1. Radio-HPLC profile of (A) HYNIC-NT(8–13), (B) MAG_3-NT(8–13) and (C) $^{99m}TcO_4^-$.

3.4. Plasma protein binding

Both ^{99m}Tc-labeled compounds bound almost equally, 22.50±1.71% and 23.08±3.55% for HYNIC-NT(8–13) and MAG3-NT(8–13), respectively.

3.5. Biodistribution studies

Table 2 summarizes the biodistribution results for both radiocomplexes. The study showed a similar pattern for both assessed radioconjugates. Quantitative analysis of data brought a better insight to the biological behaviour of different chelators (Fig. 3).

The first set of biodistribution data, 5 min pi, showed a very high uptake for blood, lung and kidneys in both cases. The %ID/ml values revealed a very fast elimination from blood for both radioconjugates with a sharp decrease after 60 min pi, where only traces of radioactivity, 0.22±0.03 and 0.26±0.10% ID/ml, can be observed for HYNIC- and MAG_3-labeled peptide, respectively. An increased liver and small intestine uptake (8.33±2.66 and 18.11±6.84 %ID/g, respectively, after 30 min pi) over time was found only for the radiocomplex labeled via MAG_3, whereas lower activity was observed for HYNIC in these organs (0.73±0.13 and 1.01±0.10 %ID/g) at the same time point.

Stomach uptake was comparatively low at all time points for both radioligands studied, indicating that there is minimal, if any, in vivo dissociation of ^{99m}Tc from these ligands to produce $^{99m}TcO_4^-$.

Following the excretion route of the radiotracer labeled via MAG_3, a noteworthy uptake was observed for the large intestine (9.06±3.52 %ID/g) at 240 min after intravenous injection, which remained high, until 360 min pi (5.60±2.02 %ID/g). No retained accumulation of radioactivity in any organ was observed at the later time point studied.

Total blood, muscle and bone are graphically expressed in Fig. 2. Both radioconjugates are quickly cleared from blood, muscle and bone, thereby reaching very low levels within 90 min pi. Although the clearance pattern of the two radiocomplexes is similar, NT(8–13) radiolabeled via MAG_3 clearly showed a much slower clearance over time for total muscle and bone as compared with NT(8–13) radiolabeled via HYNIC.

The analysis of the experimental design (Fig. 3) showed that the variables (BFCAs, organs and time) as well as their interactions were statistically significant, except for the interaction between peptide and time (P=.1938).

4. Discussion

The bifunctional chelating agent approach is currently among the cutting edge technologies used in the design of new radiopharmaceuticals. Not only because of its practical clinical application in the routine of the radiopharmacies, but also because of the high bond strength of the radiometal–biomolecule complex with subsequently high radiochemical

Table 2
Biodistribution of the radiolabeled conjugates in healthy Swiss mice

Tissue	Time (min)							
	5		30		60		90	
	HYNIC	MAG_3	HYNIC	MAG_3	HYNIC	MAG_3	HYNIC	MAG_3
%ID/g								
Blood[a]	6.83±1.20	7.41±1.28	2.11±0.46	1.22±0.35	0.22±0.03	0.26±0.10	0.06±0.02	0.08±0.02
Heart	2.45±0.34	4.80±0.98	1.12±0.34	0.55±0.16	0.11±0.03	0.23±0.16	0.06±0.01	0.13±0.06
Lung	4.44±0.62	5.22±0.51	2.08±0.40	1.41±0.31	0.34±0.11	0.55±0.20	0.16±0.03	0.41±0.17
Kidneys	24.65±4.08	28.09±7.92	10.48±5.53	10.08±4.30	5.00±0.61	5.79±2.93	3.75±1.57	5.43±0.59
Spleen	1.90±0.39	2.36±0.42	1.17±0.17	0.83±0.25	0.28±0.12	0.52±0.29	0.17±0.06	0.36±0.10
Stomach	2.44±0.50	3.55±0.38	1.16±0.14	1.23±0.22	0.40±0.15	1.11±0.47	0.25±0.07	0.55±0.30
Pancreas	1.60±0.26	3.62±1.18	0.71±0.31	0.63±0.29	0.20±0.09	0.35±0.06	0.09±0.07	0.14±0.02
Liver	1.74±0.16	17.46±1.08	0.73±0.13	8.33±2.66	0.24±0.04	6.19±2.45	0.19±0.05	5.19±1.21
Large intestine	2.52±0.54	4.61±0.44	1.57±0.13	1.39±0.38	0.35±0.12	0.99±0.62	0.22±0.16	0.92±0.61
Small intestine	1.89±0.22	4.22±0.69	1.01±0.10	18.11±6.84	0.49±0.12	18.20±5.78	0.25±0.15	11.06±4.06
Muscle	1.26±0.24	2.21±0.31	1.06±0.35	0.52±0.16	0.08±0.02	0.39±0.17	0.08±0.07	0.35±0.06
Bone	1.98±0.39	3.53±0.58	0.99±0.14	1.00±0.43	0.21±0.07	0.43±0.14	0.08±0.01	0.63±0.26
%ID								
Stomach	0.48±0.10	0.67±0.08	0.21±0.06	0.23±0.04	0.08±0.04	0.12±0.06	0.04±0.02	0.08±0.03
Kidneys	8.09±0.99	5.91±1.62	3.80±1.15	2.17±0.10	2.00±0.79	1.21±0.25	0.78±0.12	1.08±0.28
Liver	1.92±0.30	13.89±0.94	0.80±0.13	7.52±0.81	0.26±0.06	5.60±0.91	0.16±0.04	4.74±1.01
Large intestine	0.74±0.06	1.09±0.33	0.36±0.07	0.44±0.12	0.14±0.08	0.32±0.14	0.05±0.01	0.18±0.02
Small intestine	0.71±0.14	2.03±0.20	0.51±0.06	6.06±1.06	0.23±0.06	5.15±2.17	0.10±0.03	5.93±1.66

Tissue	Time (min)							
	120		240		360		1440	
	HYNIC	MAG_3	HYNIC	MAG_3	HYNIC	MAG_3	HYNIC	MAG_3
%ID/g								
Blood[a]	0.07±0.08	0.12±0.08	0.05±0.01	0.09±0.03	0.02±0.01	0.06±0.02	0.02±0.01	0.02±0.01
Heart	0.04±0.01	0.12±0.06	0.04±0.01	0.07±0.03	0.03±0.01	0.05±0.02	0.04±0.01	0.01±0.01
Lung	0.11±0.05	0.31±0.08	0.10±0.02	0.19±0.05	0.06±0.02	0.16±0.04	0.05±0.01	0.05±0.02
Kidneys	4.01±1.24	3.41±1.02	3.68±0.71	2.92±0.59	3.88±1.42	1.94±0.56	1.86±0.46	1.11±0.20
Spleen	0.16±0.07	0.27±0.08	0.21±0.04	0.32±0.10	0.12±0.05	0.24±0.06	0.12±0.07	0.22±0.02
Stomach	0.11±0.06	0.55±0.29	0.17±0.07	0.40±0.14	0.20±0.08	0.24±0.04	0.09±0.02	0.08±0.01
Pancreas	0.04±0.01	0.20±0.08	0.04±0.01	0.11±0.05	0.03±0.01	0.07±0.02	0.03±0.01	0.02±0.01
Liver	0.17±0.07	3.78±0.80	0.21±0.06	4.28±1.63	0.16±0.06	2.68±0.53	0.10±0.02	0.40±0.13
Large intestine	0.20±0.07	2.16±1.78	0.29±0.08	9.06±3.52	0.23±0.09	5.60±2.02	0.12±0.02	0.12±0.01
Small intestine	0.30±0.04	16.27±8.73	0.33±0.10	1.43±0.52	0.19±0.04	1.00±0.26	0.18±0.04	0.10±0.03
Muscle	0.03±0.01	0.31±0.18	0.02±0.01	0.12±0.04	0.02±0.01	0.10±0.04	0.05±0.02	0.01±0.01
Bone	0.09±0.08	0.28±0.09	0.12±0.02	0.22±0.09	0.08±0.03	0.14±0.03	0.08±0.03	0.08±0.01
%ID								
Stomach	0.02±0.01	0.11±0.06	0.04±0.02	0.07±0.04	0.03±0.02	0.03±0.01	0.02±0.01	0.01±0.01
Kidneys	1.42±0.47	0.78±0.27	1.46±0.51	0.78±0.13	1.28±0.36	0.42±0.12	0.65±0.15	0.26±0.01
Liver	0.17±0.17	3.23±0.36	0.22±0.05	4.19±1.99	0.16±0.05	2.14±0.61	0.14±0.03	0.41±0.08
Large intestine	0.07±0.02	0.23±0.03	0.11±0.04	2.74±0.85	0.08±0.03	0.94±0.37	0.05±0.02	0.04±0.01
Small intestine	0.15±0.02	8.33±5.60	0.15±0.07	0.90±0.55	0.12±0.04	0.33±0.12	0.09±0.03	0.01±0.01

Results are expressed as mean±S.D., n=6.
[a] %ID/ml.

yields, good in vitro and in vivo stability, and as a potentially easily accessible kit in nuclear medicine departments.

Although the choice of a chelator agent may be crucial in the biological behaviour of a radiopharmaceutical, great importance has to be given to the carrier biomolecule attached to it. In this sense, neurotensin, which is known to modulate fundamental processes in either central and periphery systems, as well as to induce growth on a variety of normal and cancer cells, such as the pancreas [23], lung [24], breast [25] and colon [19], is a putative candidate for clinical applications. However, as the majority of small peptides, if not all, native neurotensin is rapidly deactivated by peptidases in vivo [19]. Various studies, since its discovery and isolation, pointed out that the binding part is addressed to the C-terminal hexapeptide NT(8–13) [19,26]. Aside from that, finding the binding sequence is not the only drawback faced by peptides. Also, the short in vivo half-life of unmodified NT(8–13), resulting from the

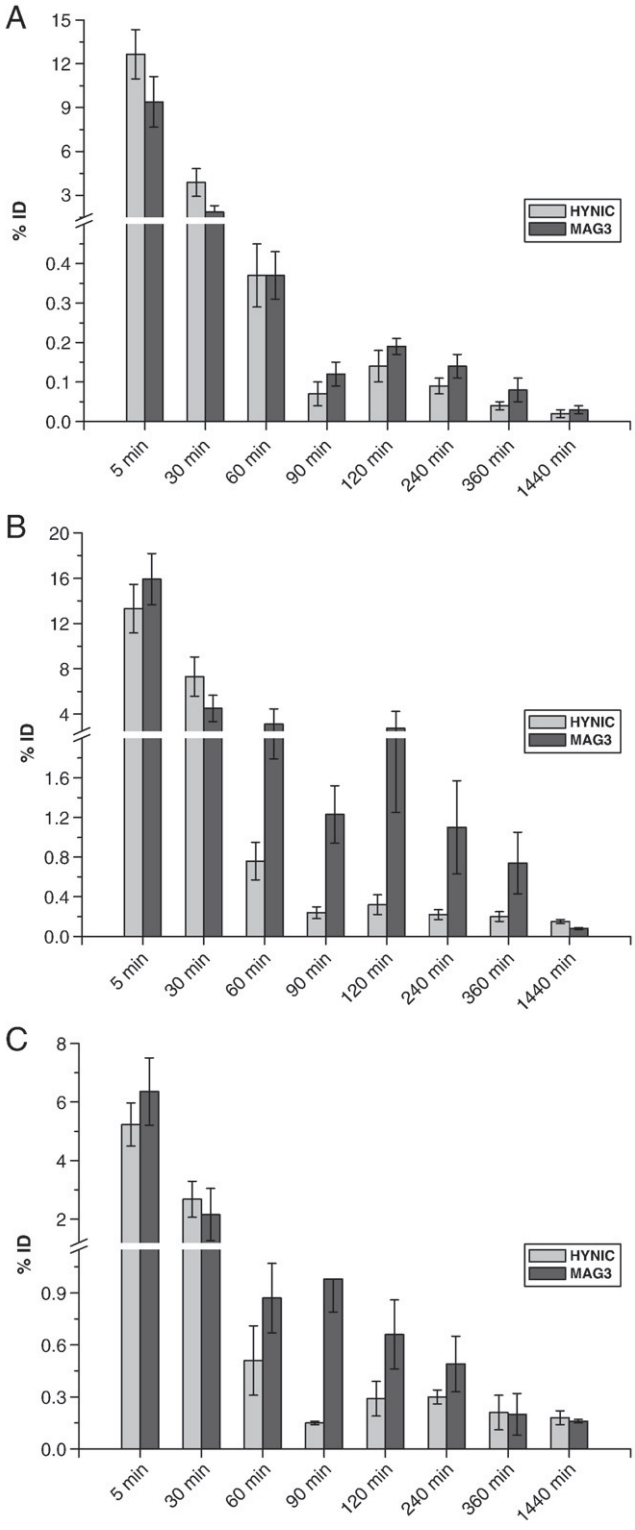


Fig. 2. Histograms of the %ID from (A) total blood, (B) total muscle and (C) total bone.

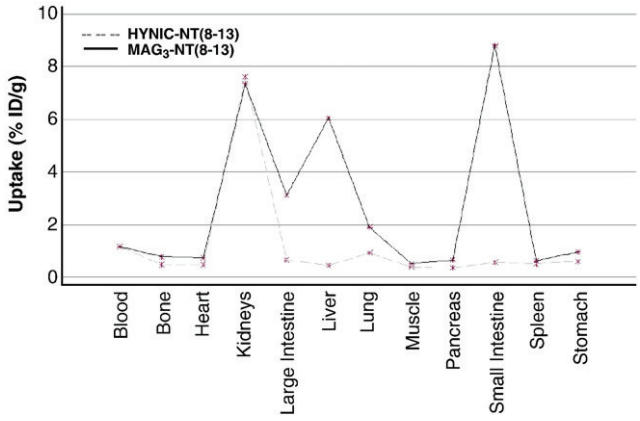


Fig. 3. Statistical analysis of the interaction plot between BFCAs (HYNIC and MAG_3) and organ uptake.

deactivation of peptidases at the cleavage sites Arg^8–Arg^9, Tyr^{11}–Ile^{12} and Ile^{12}–Leu^{13}, has been extensively explored in order to enhance its half-life as well as the in vivo stability of the radioconjugates [27,28]. Garcia-Garayoa et al. [19,27] developed a series of NT(8–13) analogues and the namely double-stabilized NT-XII is among of the most promising analogues.

The high number of isomers and the relatively low stability of ^{99m}Tc-labeled HYNIC biomolecules are still objects of investigation [6]. The only peak in the radio-HPLC profile, in the case of ^{99m}Tc-HYNIC-NT(8–13), indicated the absence of isomers. However, nothing can be ensured about the final structure of the radioconjugate. The possible exchange reactions among coligands and the possible action of HYNIC as a mono/bidentade ligand complexing ^{99m}Tc seem too far apart to be solved [6], since the complexes remain poorly characterized at the tracer level. Efforts have already been done in order to minimize the possible exchange reactions without success [29]. Otherwise, since N_3S chelators provide the four basal atoms required by the coordination chemistry of ^{99m}Tc, very stable and well-defined complexes are formed using the NHS-MAG_3 chelator [4,29].

Challenging the bioconjugates with excess cysteine or glutathione in order to determine its stability against ligand exchange and/or decomposition revealed a high stability even at the highest ratio studied for both radioconjugates. The HYNIC/tricine/EDDA system provided a very stable complex toward cysteine and glutathione transchelation. Concerning challenging experiments using cysteine as transchelator, similar results were obtained by other groups when using only tricine as coligand [4]. In contrast to our findings, a greater stability of ^{99m}Tc when radiolabeled via HYNIC vs. MAG_3 on antibodies [30] and on human serum albumin [31] has been previously reported.

Structural conformational changes caused by the BFCAs on NT(8–13)-labeled analogue seem to have no effect on plasma protein binding. We believe that this lower percentage of bound radioconjugates to plasma proteins may be due to the absence of transchelation. Similarly, no relevant differences in the percentage of protein binding were found by Vanderheyden et al. [2] when radiolabeling Annexin-V. However, high levels of the radiocomplex

bound to plasma proteins with HYNIC would be expected since studies suggested that exchange reactions of tricine coligand with proteins in plasma and lysosomes may take place [7,32].

In biodistribution studies (Table 2), a similar pattern can be observed for both radioconjugates. The major differences related to the accumulation of radioactivity may be addressed not only to the choice of the chelator, but also to the carrier biomolecule. Since the neuromodulatory actions of NT(8–13) at the periphery level are well established [16–19], a noticeable uptake in the gastrointestinal tract may be expected. The complex labeled via MAG_3 showed a very high uptake in the small intestine at 30 min pi, followed by a sharp decrease after 120 min pi. The influence of the biomolecule on these results can be eliminated by the comparison of the data obtained at the same conditions for the radiocomplex labeled via HYNIC.

A very fast elimination rate of the radioactivity from the blood and the kidneys at the earlier time points studied via HYNIC was not in accordance with that found using only tricine as coligand [33]. Furthermore, studies suggesting the use of mixed coligands (for example, tricine plus EDDA in place of only tricine) may cause a shift in the hydrophilic character of the radiopharmaceutical, consequently dropping plasma protein binding as well as increasing the elimination rate of the radioactivity by renal pathway [34].

The rapid renal excretion observed for HYNIC conjugate resulted in the lowest values in blood, liver and intestines. In the later time points studied, in the case of MAG_3, it could be observed that the main percentage of the total dose injected is accumulated in both the liver and intestines. These findings are in good agreement with partition coefficient values, showing a higher lipophilic character for the radioconjugate labeled via MAG_3 with subsequent hepatobiliar excretion when compared with the predominant renal excretion observed for the radioconjugate labeled via HYNIC.

5. Conclusion

The chemistry of HYNIC and MAG_3 as chelators is extensively reported in the literature. With this previous knowledge, the choice of the reactional parameters, coligands (in HYNIC's case) as well as protection groups (in MAG_3's case), high radiochemical yields (>97%) and an interesting comparative pharmacokinetic behaviour were obtained in the present article.

Both radioconjugates showed a similar in vitro and in vivo pattern. The results indicated that HYNIC seems to be a good choice since a very high stability as well as a predominantly renal excretion was observed. In contrast, although high radiochemical yields and a good stability of the radioconjugate labeled via MAG_3 were achieved, the remarkable liver and intestinal uptake observed even at the later time points investigated is worth mentioning, since a renal excretion is preferable for diagnostic radiopharmaceuticals. The influence on biological pattern of each BFCA was clearly highlighted in the present study and its choice would be largely dependent on the desirable application of the radiopharmaceutical. Finally, both radioconjugates are putative candidates for imaging tumors using nuclear medicine techniques.

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