REPORT OF THE COORDINATED RESEARCH PROJECT –
IAEA - CRP F22052

Establishment and standardization of a technology for ready to use production of cold kit formulation of DOTA-Rituximab and peptide based radiopharmaceuticals for labeling with lu-177 and Y-90

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IAEA's Coordinated Research Project (CRP):

Development and preclinical evaluation of therapeutic radiopharmaceuticals based on Lu-177 and Y-90 labeled monoclonal antibodies

Introduction

The purpose of this investigation was to introduce a technology for ready to use production of cold kit formulation of DOTA-Rituximab and peptide based radiopharmaceuticals (Substance P) for labeling with lu-177 and Y-90, to establish and to standardize the methods for synthesis and conjugation.

In the same time using a model system comprised in both isolated cell cultures and animal model the radiolabeled yield, biological properties and pharmacokinetic behavior of 177Lu-DOTA Rituximab and Supstance P to compare and to determine toxicities and therapeutic efficacies.

Part I

According to the proposed work plan for the first year and following the supply of the required material and chemicals (antibodies, conjugates), our work group dedicated their own activities to the Substance P derivates which have shown good potential for treatment of glioblastoma use receptors as molecular targets followed the mechanism of *Binding sites for radioligand,* where receptors are used primarily as binding sites for a peptide analog, little consideration being given to their biological function.

Substance P is a neuropeptide: an undecapeptide that functions as a neurotransmitter and as a neuromodulator. It belongs to the tachykinin neuropeptide family. Substance P and its

closely related neuropeptide neurokinin A (NKA) are produced from a polyprotein precursor after differential splicing of the preprotachykinin A gene. The deduced amino acid sequence of substance P is as follows: *Arg Pro Lys Pro Gln Gln Phe Phe Gly Leu Met.*

To obtain the peptide available for the labeling with lu-177 was necessary to start from the model already used for labeling with Tc-99m and Re-188.

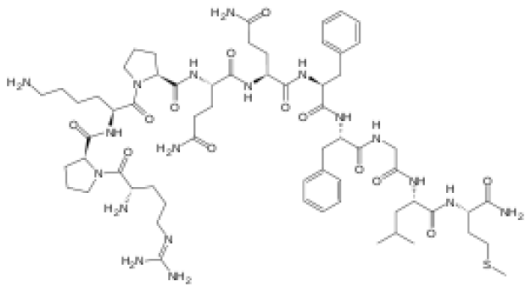


Figure 1: Substance P - Arg Pro Lys Pro Gln Gln Phe Phe Gly Leu Met

We synthesized Substance P modified for Tc-99m and Re-188 labeling and examine the characteristics including biodistribution studies in normal mice.

1. Syntesys of peptides

Peptide No.1 - Cys-Cys-SP /PCN for Tc-99m labeling

Ligands utilized for the synthesis of the new Tc-99m complex discussed here are Cys-Cys-SOST.P, [Cys-Cys-Arg-Pro-Lts-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met] and the phosphine PCN [PCN = $P(CH_2CH_2CN)_3$]. Succinic dihydrazide [SDH = H_2N-NH-C(=O)-CH_2-CH_2-C(=O)-NH-NH_2] was used in the preparations as donor of nitrido nitrogen groups (N_{3-}).
Chromatographic analysis has been carried out using HPLC methods.

[^{99m}Tc(N)(Cys-Cys-SOST.P)PCN]. 0.9mL of Na[$^{99m}TcO_4$] (100MBq) were added to a vial containing 1mg of SDH and 0.10 mg of $SnCl_2$ (suspended in 0.10 mL of saline). The mixture was kept at room temperature for 15 min. To the resulting solution was added 0.5-0.05 mg of Cys-Cys-SOST.P (dissolved in 0.5 mL of saline) and 0. 5 mg of PCN (dissolved in 0.5 mL of saline containing 2 mg of gamma-idroxipropilciclodestrin) were simoltaneously added. The vial was heated at 100° C for 30 minutes. The radiochemical yield was determined by HPLC chromatography. The final pH was 6-7.

HPLC chromatography.

Radiochemical purity was checket by:

HPLC (Beckman System) according to the following procedure:
Column Zorbax 300SB-C18, 300Anst., 5 micron (4.6 x 250 mm) with a guard column.

Detector UV and radiometric.

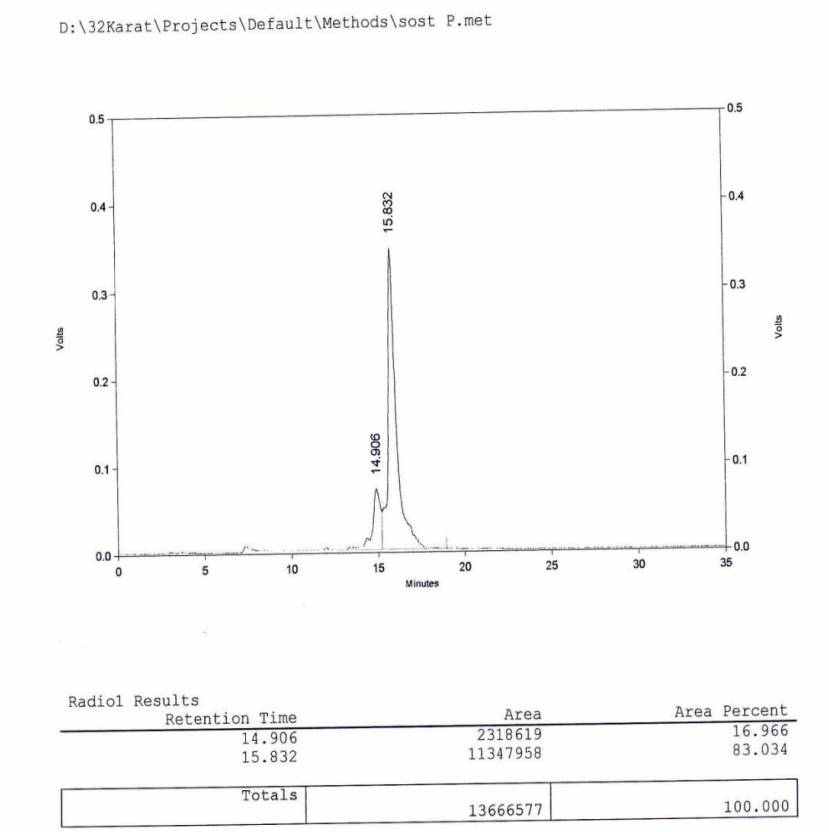
Mobil phase A: 0.1 % TFA in water

Mobil phase B: 0.1 % TFA in CH_3CN

Flow rate: 1 ml/min

Run gradient: Tr = 15.8 min.

Time (min)	% B
0 – 30	0 - 100
30 – 32	100
32 – 34	100 - 0



Radiol Results

Retention Time	Area	Area Percent
14.906	2318619	16.966
15.832	11347958	83.034
Totals	13666577	100.000

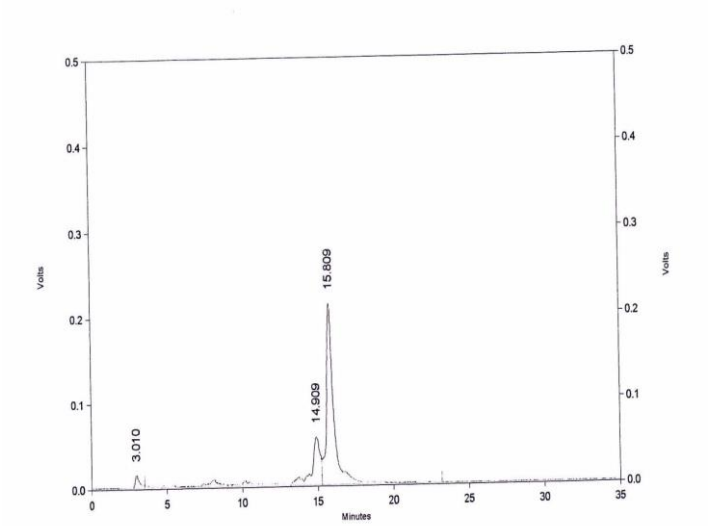
Figure 2: HPLC chromatogram

Stability - Cysteine Challenge

250.0 ul of a phosphate buffer solution (0.20 M, pH = 7.4), 50.0 uL of a freshly prepared solution of cystein (10.0 mM in a water solution), 100.0 uL of water and 100.0 uL of the Tc-99m complex were placed in a polypropylene tube and incubated in a water bath, at 37°C. For the blank an equal volume of water is added instead of cysteine solution. Aliquots of the

resulting solutions were withdrawn at 60 min after incubation, and analyzed by HPLC chromatography.

The complex was found to be stable against transchelation by cysteine



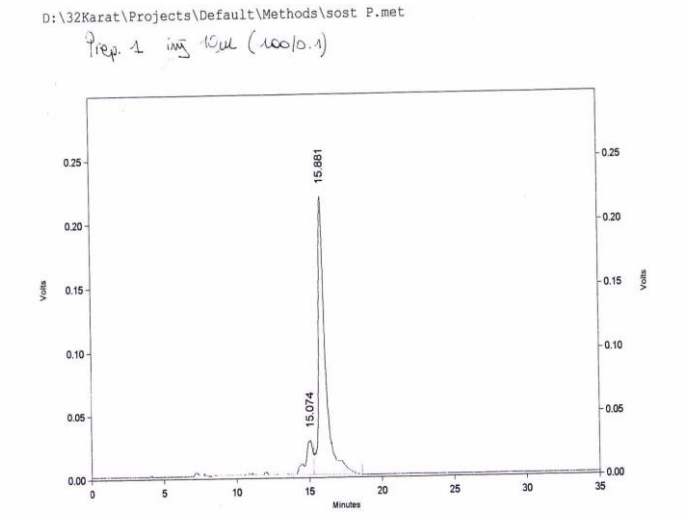
Radiol Results

Retention Time	Area	Area Percent
3.010	290101	3.016
14.909	2177804	22.643
15.809	7149931	74.340
Totals	9617836	100.000

Figure 3: The complex was found to be stable against transchelation by cysteine

In vitro reaction with glutathion (GSH). 250.0 ul of a phosphate buffer solution (0.20 M, pH = 7.4), 50.0 uL of a freshly prepared solution of GSH (10.0 mM in a water solution),, 100.0 uL of water and 100.0 uL of the Tc-99m complex were placed in a polypropylene tube and incubated in a water bath, at 37°C. For the blank an equal volume of water is added instead of GSH solution. Aliquots of the resulting solutions were withdrawn at 60 min after incubation, and analyzed by HPLC chromatography.

Serum stability. 225.0 ul of serum and 25.0 ul of the Tc-99m complex were placed in a polypropylene tube and incubated in a water bath, at 37°C. Aliquots of the resulting solutions were withdrawn at 30, 60 and 120 min after incubation, and analyzed by HPLC chromatography.

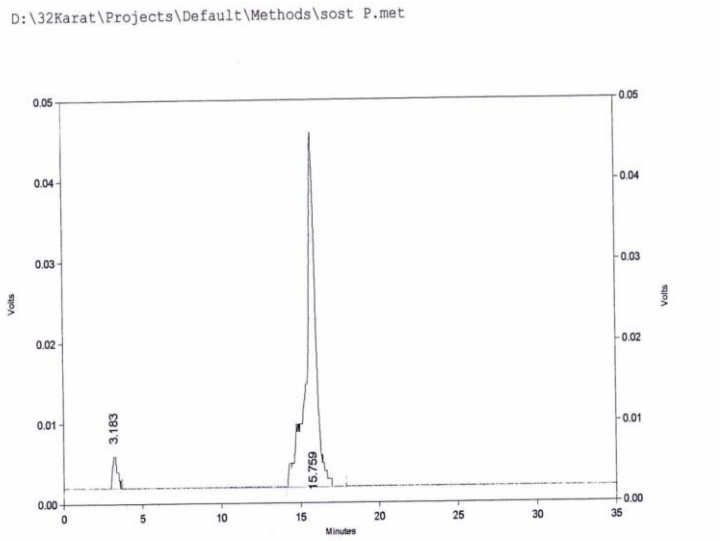


Radiol Results

Retention Time	Area	Area Percent
15.074	934003	11.581
15.881	7130760	88.419
Totals	8064763	100.000

Figure 4: The complex was found to be stable.

The evaluation of the serum stability was made using rat and human serum



Radiol Results

Retention Time	Area	Area Percent
3.183	84728	4.512
15.759	1793013	95.488
Totals	1877741	100.000

Figure 5: Stability in the human serum 1hour min at 37$_0$C

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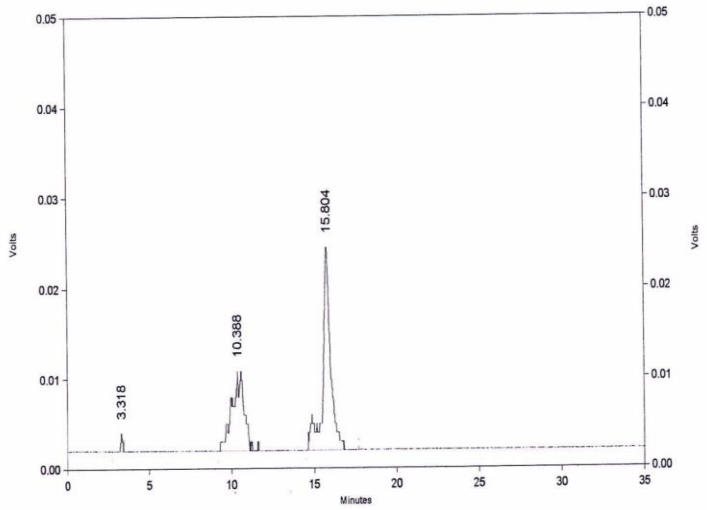

Radio1 Results

Retention Time	Area	Area Percent
3.318	14111	1.199
10.388	439291	37.319
15.804	723725	61.482
Totals	1177127	100.000

Figure 6: Stability in the rat serum 30 min at 37^{0}C

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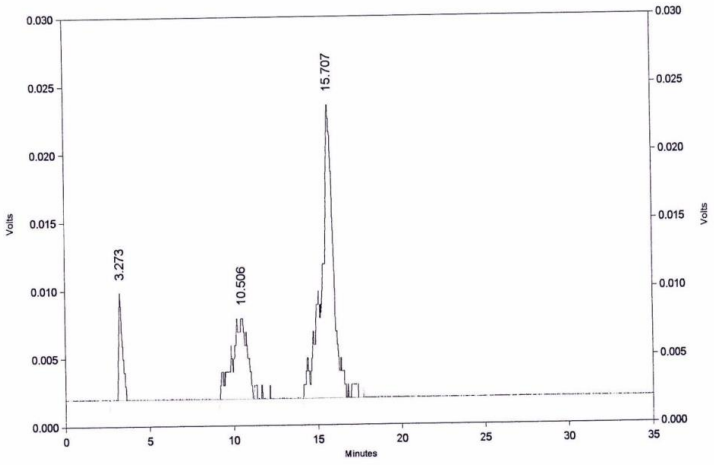
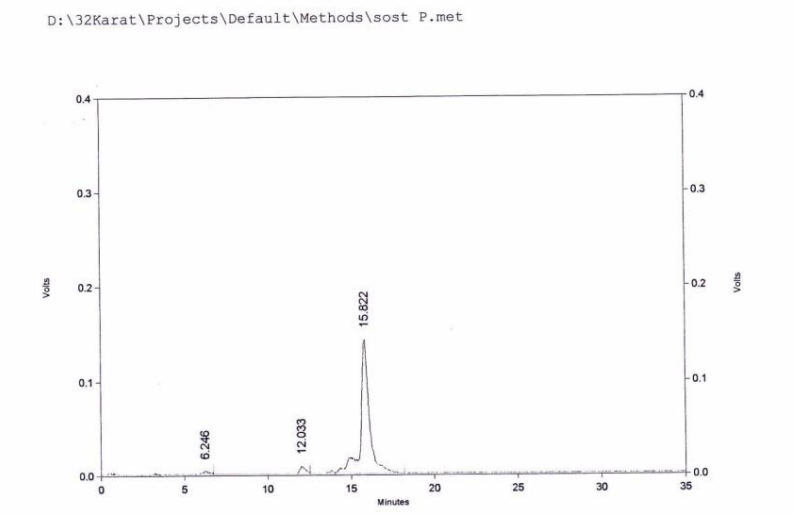


Radio1 Results

Retention Time	Area	Area Percent
3.273	115174	7.494
10.506	364013	23.685
15.707	1057702	68.821
Totals	1536889	100.000

Figure 7: Stability in the rat serum 1hour min at 37^{0}C

We find that in rat serum the complex is not stable starting from 30 min and in human serum is stable at 30 min but not stable starting from 60 min.
The “in vivo” stability you can see the chromatograms of the 14/07 when we worked with mice. The obtained complex was stable after 24 hour



Radiol Results

Retention Time	Area	Area Percent
6.246	78680	1.462
12.033	168058	3.124
15.822	5133524	95.414
Totals	5380262	100.000

Figure 8: Stability of the complex after 24 hours in human serum

Peptide No.2 - Cys-Cys-SP /PCN for Re-188 labeling

Introduction. Ligand utilized for the synthesis of the new Re-188. Chromatographic analysis has been carried out using HPLC methods.

[^{188}Re(O)(Cys-Cys-SOST.P)]. 1.8mL of Na[^{188}ReO$_4$] (100MBq) were added to a vial containing 1.0 mg of $SnCl_2$, 5 mg of ascorbic acid, 28 mg of sodium oxalate and 0.1 mL of acetic acid glacial. The mixture was kept at 100°C for 1 hour.
The radiochemical yield was determined by HPLC chromatography. The final pH was 3.

HPLC chromatography. Radiochemical purity was checket by:

HPLC (Beckman System) according to the following procedure:

Column Zorbax 300SB-C18, 300Anst., 5 micron (4.6 x 250 mm) with a guard column.

Detector UV and radiometric.

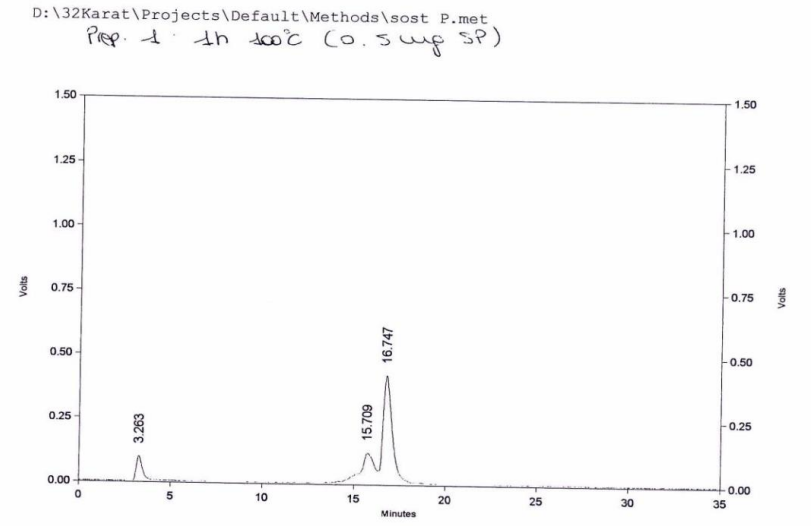
Mobil phase A: 0.1 % TFA in water

Mobil phase B: 0.1 % TFA in CH_3CN

Flow rate: 1 ml/min

Run gradient: Tr = 15.8 min.

Time (min)	**% B**
0 – 30	**0 - 100**
30 – 32	**100**
32 – 34	**100 - 0**



Radio1 Results

Retention Time	Area	Area Percent
3.263	2194409	9.007
15.709	6690480	27.460
16.747	15479427	63.533
Totals	24364316	100.000

Figure 9: HPLC chromatogram

2. SPECT studies

The synthesized Substance P was labeled with Technetium -99m and injected in to the mice for normal distribution studies.

SPECT data acquisition

The SPECT studies have been performed by using the YAP-(S)PET scanner [1]. A mice (~25 g) was injected with 75 MBq (2mCi) of [99_mTc]-substance P to study the tracer whole-body distribution. The mice was scanned the first time for 60 min starting 20 min after injection . The SPECT acquisition consists of 3 bed position 3 cm apart, 20 min per bed position, 128 views over 360°. The second acquisition starts 1h 30min after the injection with the same modalities of previous one. The used energy window is 140-250 keV and the images were reconstructed by using the iterative EM-ML algorithm including the collimator response.

X-ray data acquisition

The radiographic images have been acquired by using the integrated digital X-ray imaging system of YAP-(S)PET scanner [2]. The acquisition parameters for the x-ray projections were: x-ray tube voltage 35kVp, anode current 1mA, exposure 1 s, 8 views over 360° and magnification factor 1.2. Each final image have been obtained by subtracting dark noise contribution and performing flat field correction.

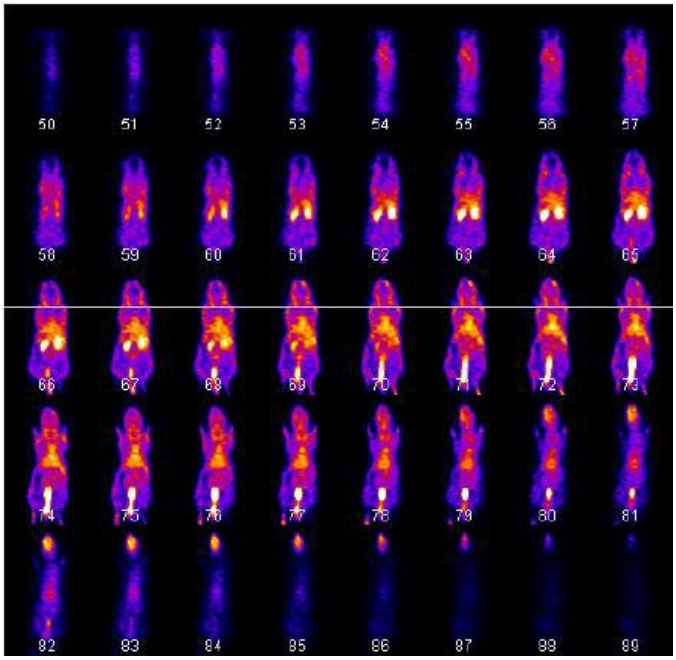


Figure 10 : Mice 140711_140026 coronal sections – Starting at 20min from the injection

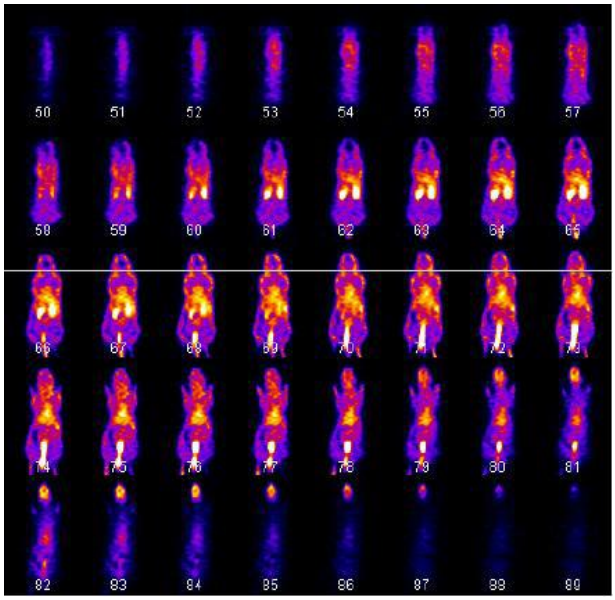


Figure 11 : Mice 140711_151532 coronal sections – Starting at 1h34min from the injection

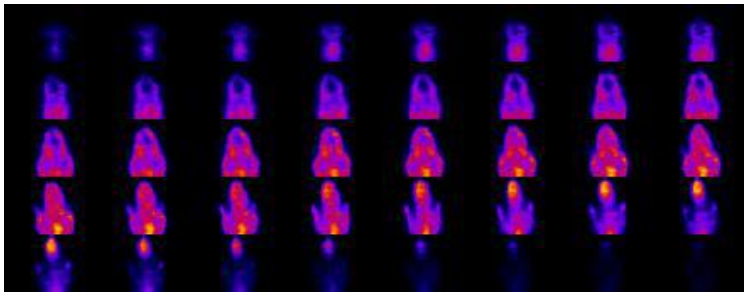


Figure 12: Mice 140711_162959 coronal sections – Starting at 2h50min from the injection

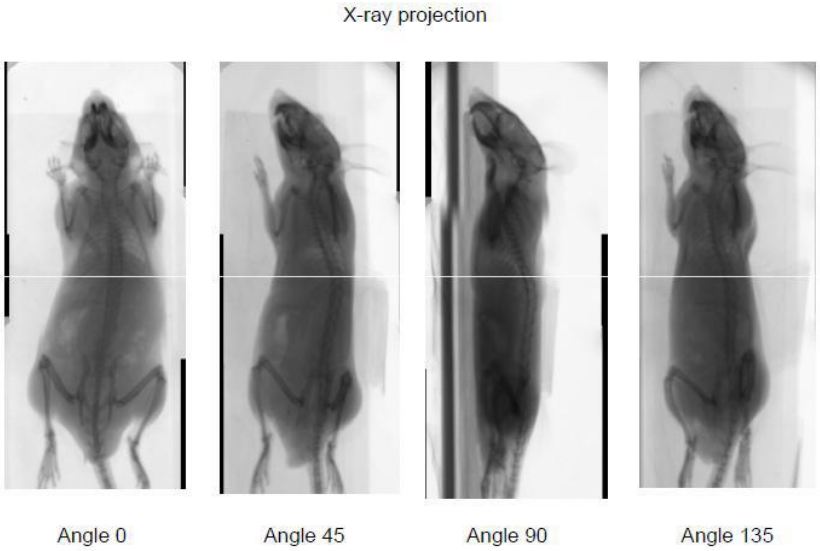


Figure 13: Mice in X – ray projection

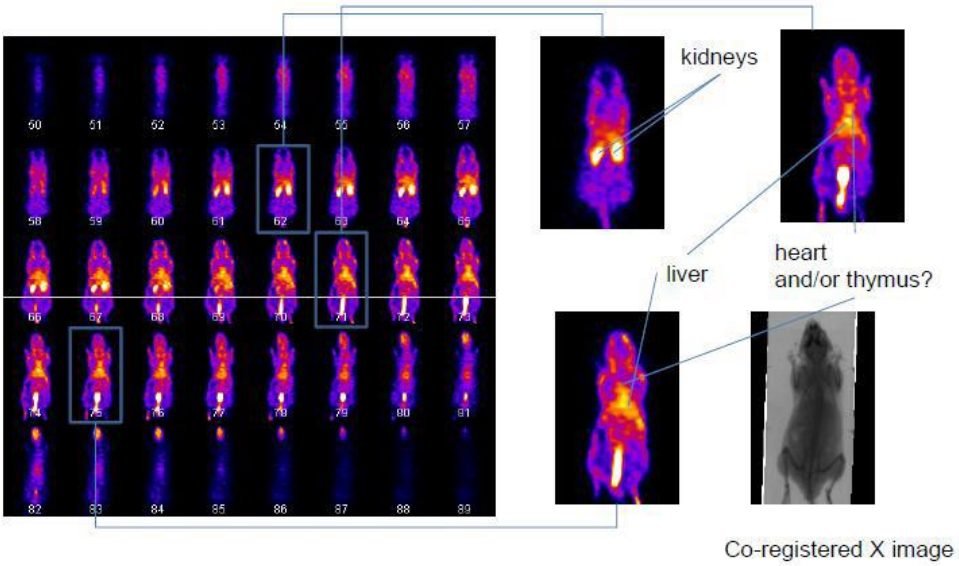


Figure 14: Mice 140711_140026 coronal sections – Starting 20min from the injection

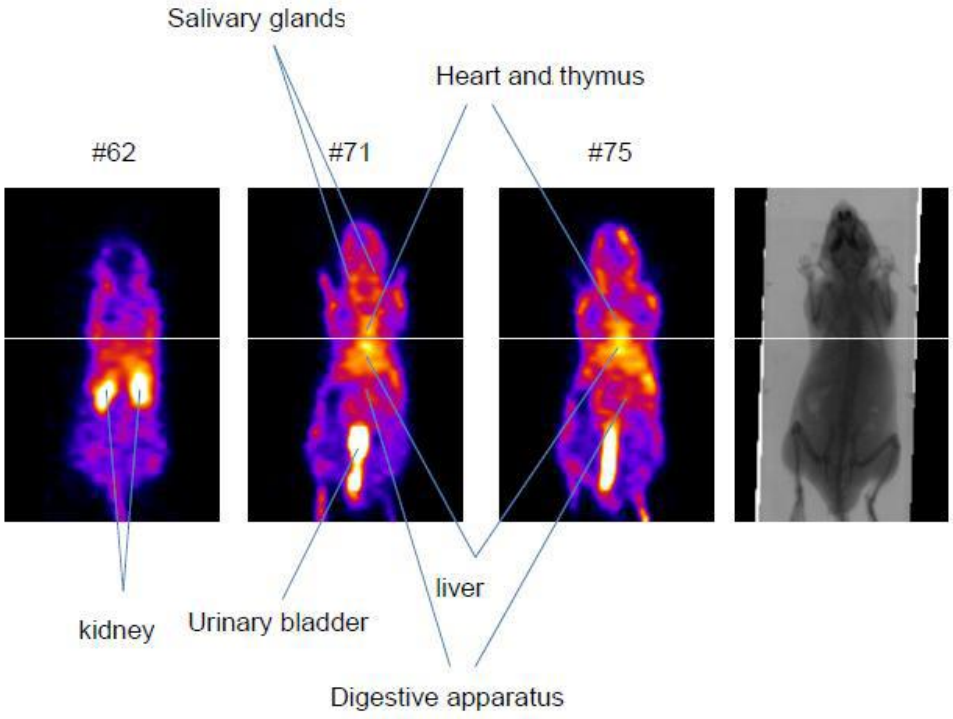


Figure 15 : The comparison of SPECT – CT distribution

Conclusion:

The obtained results show that the synthesized Substance P is stable and can be labeled used radioactive isotopes.
Our results are obtained using already standardized method of labeling with Technetium-99m and renium-188 to show the stability of the labeled product .
The normal animal distribution contribute to the normal localization of the Substance P:

- Kidneys and urinary bladder have high activity concentration;
- Liver is with a good activity concentration),
- digestive apparatus with the trace of the activity
- localized radioactive distribution in the heart and thymus
- and the localization in the salivary glands.

The imaging study of the normal distribution in the normal mice can be good indicator for the next experiment in which we will synthesize the Substance P available for labeling with Lutetium-177.

Part II

Introducing protocol for freeze drying of antibodies

The other part of our work is to establish the procedure of freeze drying to obtain the final kit formulation for the antibody, ready for labeling.
The procedure of freeze drying should provide stable formulation identically available for preclinical investigation as the freshly prepared solution. The freeze dried formulation should have the same immunoreactivity of the conjugated antibody before conjugation and after conjugation in the liquid formulation.
For this reason the most important steps in which we were working in our first part of the project was to establish the protocol and to estimat the Freeze-Drying Time for Lyophilized Formulations of Monoclonal Antibody in each phase – Prefreezing, Primary Drying and Secondary Drying.
In the same time we were working how to provide formulations of high concentration proteins, such as monoclonal antibodies, Immunoglobulins (IgG) and to define the freeze-drying process, i.e., freezing temperature and drying time,

Product Quality Concerns and Issues

The final product was examined on:

- Appearance
- Specific Surface Area
- Sublimation Rate
- Residual Moisture and Recon Time
- Physical State of Protein and Excipients in the Cake
- Crystallinity
- Protein Aggregation, Changes in Conformation and Bioactivity
- Stability over Time

Freeze Drying Procedures

The freeze drying protocol was introduced using the procedures used for BSA, IgG, mAb (Mabthera) following:

- Volumne of solution – 1ml
- Filled into 2ml glass vial (fill depth = 0.75 cm)
- Freeze Drier LABCONCO

Initial procedure

- Ramp from room temperature to -45^{0}C , (ramp rate 1^{0}C/min)
- Hold for 2h, ramp to -20^{0}C (1^{0}C/min)
- Hold for 1h and return to -45^{0}C
- Maintain shelf temperature for 2 hours
- Primary drying was conducted at chamber pressure (Pc) of 57 mTorr and shelf temperature of -25^{0}C and +25^{0}C
- Chamber pressure (Pc) was constant for primary and secondary drying
- Prymary drying for BSA was 4 min and 3 min for IgG and mAb
- Secondary drying
- Shelf temperature of 40^{0}C for 10 hours (increase 15-20 hours) - (ramp rate 1^{0}C/min)

Results:

The stability of the freeze dried products was measured for:

- IgG – 6 month used freeze dried formulation
- BSA – 10 month
- mAb (Mabthera) – chek only for 3 months

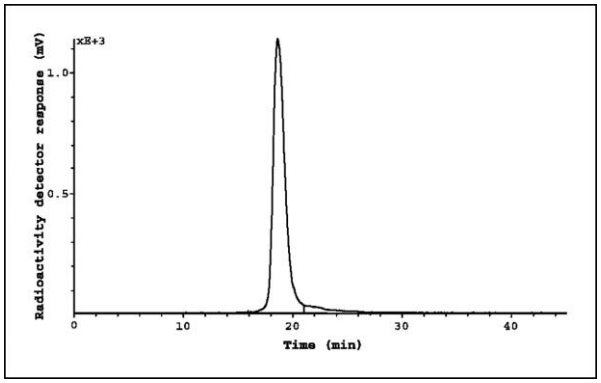


Figure 16 : Freeze dried BSA – 10 months stability using HPLC (radiation detection) and labelled with 125 Iodine

The next work will be dedicate to reproduce the procedure of freeze-drying in the new freeze-drier 24 and 48 hours using anti-CD (Mabthera – commercial available – Roche)

- To check the solubility with visible control
- Using Electron Microscope and to check the structure and size of the freeze dries molecule ad
- Immunoreactivity of freeze dried conjugate in comparison with liquid