REPORT

on the

2\textsuperscript{nd} Research Coordination Meeting

on

‘The Development of Therapeutic Radiopharmaceuticals Based on \( { }^{188}\text{Re} \) and \( { }^{90}\text{Y} \) for Radionuclide Therapy’

22 - 26 March 2010

Vienna, Austria
2nd Research Coordination Meeting

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BACKGROUND

Radionuclide therapy is practiced for the treatment of malignant disorders of various organs and tissues as well as for treating certain other diseases such as rheumatoid arthritis. Advances in understanding tumor biology as well as developments in peptide chemistry and monoclonal antibody technology are opening new opportunities for the development of therapeutic radiopharmaceuticals, thereby widening the scope of radionuclide therapy. In addition, particulate based radiopharmaceuticals are useful for treating hepatocarcinoma as well as in radiation synovectomy.

With the establishment of new products the demand and application of therapeutic nuclear medicine is expected to grow rapidly. While there are a large number of radioisotopes proposed for targeted therapy, practical considerations had been limiting the number of usable isotopes. Generator-produced radionuclides are an attractive option for the large scale on-site availability of therapeutic isotopes. The IAEA’s CRP on the ‘Development of generator technologies for therapeutic radionuclides’ (2004-2007) was successful in developing technologies for the preparation of $^{188}$W/$^{188}$Re and $^{90}$Sr/$^{90}$Y generators for eluting $^{188}$Re and $^{90}$Y of high radionuclidic and chemical purity usable for research applications in the development of therapeutic radiopharmaceuticals.

The IAEA’s CRP on ‘The development of therapeutic radiopharmaceuticals based on $^{188}$Re and $^{90}$Y for radionuclide therapy’ was formulated to focus on enhancing the capacity of the $^{90}$Sr/$^{90}$Y generator; to develop and validate quality control methods for the generator eluate; and to develop therapeutic radiopharmaceuticals based on $^{188}$Re and $^{90}$Y.

The first RCM of the CRP was held in Polatom, Warsaw, Poland from 30 June to 4 July 2008. The meeting reviewed the work going on in the different participating laboratories, and the facilities, expertise and capabilities of the different participating groups, and formulated the work plan of the CRP. The work plan for the first 18 months of the CRP had three main components, which were split into several subprojects. The meeting report which was prepared post RCM and circulated among the participants gives details about the projects identified to be implemented during the CRP, the rationale for identification of the projects and a detailed work plan. During the RCM, subproject coordinators were identified with responsibility to liaise among the participating groups and monitor the progress of each sub project. An overall coordinator of the CRP was designated, whose responsibility was to liaise with the subproject coordinators. The overall coordinator was also to make six monthly reports on the progress of the CRP. The two six monthly reports thus prepared were circulated among all the CRP participants in order to disseminate the details of the work done in each laboratory.

The second RCM of the CRP was held from 22 to 26 March 2010 at IAEA Head Quarters in Vienna. The purpose of the meeting was to review the progress of the work done in the first 18 months; to make necessary mid term corrections on the CRP’s implementation and to formulate the work plan for the second half of the CRP. This report gives the summary of the work done in each participating laboratory, the overall achievements in the first 18 months of the CRP, the work plan for the second part of the CRP as well as recommendations to the IAEA, detailing the participants’ inputs, for the implementation of the CRP.
OVERALL OBJECTIVE

The overall objective of the CRP is linked to the objective of Project 2.5.1.3 (2008-9): Cost effective radiopharmaceuticals development. The objective of the project is to find radioisotopic techniques based solutions to specific clinical needs of the developing world in the area of cancer treatment, through the development and application of locally produced radiopharmaceuticals.

SPECIFIC RESEARCH OBJECTIVE

To develop radiopharmaceuticals for targeted therapy using $^{188}$Re and $^{90}$Y and to study the performance of generators with long lived parent radionuclides as well as to validate the QC control procedures for estimating the purity of generator eluates.

EXPECTED RESEARCH OUTPUTS (RESULTS)

- $^{188}$W/$^{188}$Re generators prepared using imported high specific activity $^{188}$W.

- High specific activity $^{188}$Re labelled molecules for targeted therapy.

- $^{90}$Y from a $^{90}$Sr/$^{90}$Y generator based on one or more of the technologies such as extraction chromatography, supported liquid membrane or electrochemical separation.

- Validated analytical technique based on extraction paper chromatography for the estimation of ppm levels of $^{90}$Sr in $^{90}$Y solution.

- Preclinical evaluation of at least two products based on $^{90}$Y and/or $^{188}$Re.

- Scientific data for the preparation of several publications describing the preparation novel $^{188}$Re and $^{90}$Y generators and radiopharmaceuticals.

- A Technical Report Series (TRS) publication containing the work done and results achieved through the CRP.

SUMMARY OF COUNTRY PRESENTATIONS

BRAZIL

The overall objective of this CRP is to develop radiopharmaceuticals for targeted therapy using $^{188}$Re and $^{90}$Y and to study the performance of generators with long lived parent radionuclides as well as to validate the QC control procedures for estimating the purity of generator eluents. The CRP is expected to enhance the capability in production of $^{90}$Y and $^{188}$Re radiopharmaceuticals to meet the increasing demand of therapeutic products for clinical applications, in particular in Brazil. In this period, efforts were made towards the assembling of $^{90}$Sr-$^{90}$Y generators, quality control of $^{90}$Y, the labelling of DMSA (V) and anti-CD20 with $^{188}$Re and the labelling of hydroxipatite(HA) with $^{90}$Y.

$^{90}$Sr/$^{90}$Y Generators

Two generators were prepared based on cation exchange resins, following the work from the previous CRP. The resin employed was Dowex 50W-X8 (100-200 mesh), H⁺ form. The elutions are being followed up to 7 months.
Cold and tracer studies were performed with the electrochemical generator, suitable for learning the technique.

Radionuclidic quality control procedures of Y-90 were developed, in particular using ICP-OES and liquid scintillation counter.

*Labelled molecules with $^{188}$Re*

Extensive studies were performed concerning the labeling of anti-CD20 and DMSA(V) with Re-188. Quality control methodologies were developed and optimized formulations were obtained.

*Routine production of $^{90}$Y-HA*

IPEN has a program of producing colloids for radiation synovectomy and among the products is $^{90}$Y-HA. The labelling yields > 80% with radiochemical purity higher than 98% could be obtained. The mean particle size is 15 μm. The radioactive concentration is 450-550 MBq/mL.

**CUBA**

During the first period of this CRP, we could test an efficient and reliable generator system based on ion-chromatography to obtain $^{90}$Y from its parent radionuclide $^{90}$Sr. This production scheme for $^{90}$Y was outlined in the previous CRP related with the development of generator technologies. Quality parameters such as trace metals that can potentially interfere in the labeling of biomolecules, $^{90}$Y recovery, $^{90}$Sr/$^{90}$Y ratio and radiation dose to bed matrix were evaluated. The results showed that high recovery and radionuclidic purity could be obtained for $^{90}$Y during its repeated separation from the $^{90}$Sr generator. No replacement or treatment of the generator was necessary and low waste generation and $^{90}$Sr losses less that 0.1% after each run were also observed during the present study.

A Fab’ fragment was enzymatically produced and purified from the monoclonal antibody h-R3 (Nimotuzumab®). The fragment and the parent antibody were successfully conjugated with DOTA and labeled with $^{90}$Y. The radioimmunoconjugate thus obtained also exhibited good 24 h *in-vitro* stability in an excess of DTPA. A $^{90}$Y radiocolloid was prepared in a chromic phosphate particle for radiosynoviotherapy with promising results in animal models. Two alumina based $^{188}$W/$^{188}$Re generators were prepared and their eluates were used in the labeling of hR3-DOTA conjugates. Quality control and *in vivo* evaluation in comparison with $^{99}$mTc-hR3 showed very good results and similar pattern of distribution and pharmacokinetic and will be used in clinical trials for cancer patients.

**GERMANY**

The vectors to be used to transport radionuclides into tumor tissue for treatment are antibodies against lymphomas and neuroblastomas. The antibody against Non-Hodgkins Lymphoma (NHL) can be acquired commercially and then modified for binding to the therapeutically active nuclide, $^{90}$Y. The antibody against is home-made and only available in Cologne. Neuroblastoma antibody also need to be modified to bind to Y-90 but is produced in Cologne. To improve the therapeutic value of antibodies we tried to introduce the pretargeting method.

Briefly, the first component of a three step pretargeting strategy consists of the biotinylated antibody. This includes the protocol for determination of the number of biotin molecules per antibody. By using this technique one can have a stock of biotinylated antibody in lyophilised
form ready for further experiments. In the second step, commercially available avidin/streptavidin will be used. Third and final step is the binding of radiolabelled (\(^{90}\)Y, \(^{188}\)Re) biotin to the tumour cells through the avidin-antibody-bridge after administration of a clearing agent.

Initial evaluations of the potential radiopharmaceuticals has been carried out by in-vitro experiments on cell lines expressing the corresponding antigen.

Work done so far for three-step pretargeting method can be summarized as follows:

- \(^{90}\)Y-labelling of Biotin-DOTA
- Coupling of biotinylated Rituximab to CD20-positive Raji-cells
- Successful labelling of cells conjugated with a complex of biotinylated antibody and avidin with \(^{90}\)Y-DOTA-Biotin
- Surviving of a long-lasting permission procedure for animal experiments file number 8.87-51.04.20.09.339 (25th January 2010, 134 mice)

Furthermore we achieved some new results with I-131 and Y-90 labelled ERIC1 antibodies, directed against NCAM-positive neuroblastomas. The radioiodine labelled anti-NCAM antibody ERIC1 seems to be a promising radiopharmaceutical for treatment of neuroblastoma. The optimal dose is 1-2 MBq/animal. This would correspond to about 600-1200 MBq in humans (for a child of 15 kg). First experiments with \(^{90}\)Y-labelled ERIC1 have been started and are very promising. These antibodies showed an unequivocal therapeutic effect, whereas I-131-MIBG showed no influence on the tumour growth on much higher radioactivities applied per animal. Our plans for the future are:

- Improved dose-finding studies
- Fractionation of radiation dose realized by i.v. application of radiolabelled ERIC1
- Investigation of other radioimmuno-constructs directed against NCAM
- Investigation of other tumour types with NCAM on tumour cells
- Pre-targeting

INDIA

During the first half of the CRP, the following work was carried out:

Validation of an extraction paper chromatography (EPC) technique for determination of \(^{90}\)Sr contamination in \(^{90}\)Y eluates from generator systems

The EPC technique involves the impregnation of 2-ethyl hexyl, 2-ethyl hexyl phosphonic acid (KSM-17), an \(^{90}\)Y-specific chelate at the origin (R_f=0) of a Whatman 3 mm chromatography paper. \(^{90}\)Sr which moves to the solvent front is estimated by counting using a liquid scintillation counter. The EPC technique was compared with the US Pharmacopeia recommended method for \(^{90}\)Sr contamination in \(^{90}\)Y samples. The method was validated by analyzing >2 months decayed \(^{90}\)Y samples for \(^{90}\)Sr content, to ascertain estimation of equal amounts of \(^{90}\)Y and \(^{90}\)Sr as well as by determining the recovery of doped \(^{85+89}\)Sr in \(^{90}\)Y,
linearity of response with dilution etc. \(^{90}\)Y samples eluted from Supported Liquid Membrane (SLM) based and electrochemical \(^{90}\)Sr/\(^{90}\)Y generators were analyzed using the EPC method and the \(^{90}\)Sr levels were determined to be much less than the prescribed limits.

**Development of radiolabeled anti CD20 antibodies (Rituximab) for therapy of Non-Hodgkin’s Lymphoma (NHL)**

Towards the aim of indigenous development of radiolabeled antibodies for cancer therapy, Rituximab (anti CD20 antibody) was radiolabeled with \(^{131}\)I for therapy of NHL. *In vitro* cell uptake studies of \(^{131}\)I labeled anti CD20 antibody was carried out in Raji and Daudi cells expressing CD20 antigen on the surface. Further *in vivo* biological evaluation in animal models of lymphoma is planned. Towards the development of \(^{90}\)Y labeled anti CD 20 antibody, p-NCS-Bz-DOTA was conjugated to anti CD20 antibody and radiolabeling was carried out with \(^{90}\)Y. The product was purified using PD-10 column and characterized by HPLC. Further *in vitro* is planned.

**Biological evaluation of \(^{90}\)Y oxine in lipiodol for liver cancer therapy**

In order to determine the potential of \(^{90}\)Y oxine dispersed in lipiodol for liver cancer therapy, *in vitro* studies in liver cancer cell lines and *in vivo* studies in animal model of liver cancer was carried out. Various *in vitro* assays were carried out in HepG2 human liver carcinoma cell line to determine the effect of \(^{90}\)Y activity on the cells. *In vivo* biodistribution studies were carried out in Wister rats induced with liver cancer by chemical carcinogenesis. The biodistribution studies showed significant leakage of the preparation from the liver and the subsequent localization of \(^{90}\)Y in the bone which is of serious concern. **ITALY (Chinol)**

A novel biotin-DOTA conjugate (ST2210: reduced biotinamidohexylamine-DOTA) was investigated in order to collect all the necessary data for its full scale clinical application in the newly developed therapy called IART\(^\circledast\): Intra-operative Avidination for Radionuclide Therapy. \(^{90}\)Y and \(^{177}\)Lu were used to label ST2210. The effect of pH on radiolabeling and a wide range of specific activity (SA) were studied. Radiolabeled ST2210 was tested for the affinity towards avidin and for the stability in saline or in human serum with and without ascorbic acid (AA) as radical scavenger. High radiochemical purity RCPs (>99%) were routinely achieved by reacting, at 95°C for 30 min, ST2210 with \(^{90}\)Y or \(^{177}\)Lu in sodium acetate buffer (1.0 M, pH 5.0) at SA of 2.6 MBq/nmol. Both \(^{90}\)Y- and \(^{177}\)Lu-ST2210 were also pre pared at higher SAs of 5.3 and 1.3×10\(^2\) MBq/nmol, respectively, with RCP >99% to improve its spectrum of clinical applications. Radiolabeled ST2210 was stable up to 96 h in human serum and saline with the addition of AA. The structural modifications proposed for the ST2210 such as the lack of the amide target site for the serum biotinidase, stabilized it towards the enzymatic degradation while retaining high binding affinity towards avidin. These encouraging preclinical results and the initial clinical observations obtained with this new biotin-DOTA conjugate raised the hope for the clinical success of IART\(^\circledast\). Enhancements of the therapeutic potential of this approach will be evaluated with respect to the delivery of higher radiation dose to the tumour. In this frame, the next 18 months of the CRP will be devoted to evaluate newly synthesized biotin derivatives, carrying two DOTA groups per molecule which can bind up to two metallic radionuclides.

**ITALY (Duatti)**

A new bifunctional ligand for conjugation of biotin to the therapeutic radionuclide Re-188 has been synthesized and used for the preparation of a series of \(^{188}\)Re-nitrido complexes having
interest for their potential application to the IART (Intraoperative Avidination for Radionuclide Therapy) treatment of residual post-surgical breast cancer. To meet current regulatory requirements for further biological and clinical evaluation of these new agents, we developed a freeze-dried kit formulation for their in-hospital preparation. In this attempt, the main challenge came from the difficulty to find an effective donor of nitride nitrogen groups (N$_3^-$) suitable for undergoing all steps of the lyophilization process without damage of its donor ability. Surprisingly, the compound SDH (succinic dihydrizide), which is commonly used in kit formulations for preparing the $^{99m}$Tc-nitrido core, gave poor and not fully reproducible results. Thus, we turned to substituted derivatives of dithiocarbazic acid [DTCZ = H$_2$N-N(R)-C(=S)SR'] as it was previously demonstrated that this reagents are among the strongest N$_3^-$—donors for Tc-$^{99m}$ and Re-$^{188}$. However, since DTCZ is also a strong chelating ligand for these metals, a fact that could prevent its subsequent replacement by other biologically relevant molecules, we prepared a substituted derivative of DTCZ where a sterically encumbering polyethylene glycol (PEG) chain was appended to the terminal amino group. This modification dramatically decreased the chelating properties of DTCZ while keeping almost unaltered its donor properties. Using this new reagent, we developed a two-vial kit formulation that are currently under evaluation for testing its stability, but that was found to allow the preparation of the new Re-$^{188}$ biotinylated compounds with high specific activity starting from generator-eluted $[^{188}\text{ReO}_4]^-$. 

KOREA

The $^{99m}$Tc(I) and $^{188}$Re(I) tricarbonyl precursors $[\text{M(\text{OH}_2)\text{3(CO)}_3}]^+$ have been shown to be excellent starting materials for the synthesis of further $^{99m}$Tc(I) and $^{188}$Re(I) tricarbonyl complexes as well as radiolabeling of target specific biomolecules. The tricarbonyl precursor is an attractive core for the introduction of $^{99m}$Tc and $^{188}$Re into biomolecules because of its high chemical stability and small size. Recently, a user-friendly kit formulation (IsoLink™) was developed using potassium boranocarbonate, K$_2[\text{BH}_3\text{CO}_2]$, for the preparation of the $^{99m}$Tc precursor complex. This solid reagent serves both as a source of carbon monoxide and a reducing agent for technetium. It was also used by Schibli and co-workers for the preparation of the corresponding $^{188}$Re precursor complex. The preparations resulted in yields >85 % of the desired precursor complex, remaining perrhenate (7±3 %), colloidal $^{188}\text{ReO}_2$ (<5 %), and a by-product of unknown composition. To overcome the moderate yields, a improved method for $^{188}$Re(I) tricarbonyl precursor $[^{188}\text{Re(OH}_2)\text{3(CO)}_3]^+$ was established by using borohydride exchange resin (BER) as an additional reducing agent and anion scavenger. The applicability of the newly developed method was examined in aspects of stability, safety, specific activity, etc. Further, characterization and demonstration of the method is being performed for therapeutic radiopharmaceuticals in collaboration with the participating research groups. Development of novel $^{188}$Re-radiopharmaceuticals functioning as a targeted therapeutic agent and in vivo imaging agent is currently being performed. In addition, development of quality control techniques by using various analytical instruments will be proposed along with the main research activities.

PAKISTAN

Production of Strontium-85 and yttrium-90 was carried out inside the core of the 10 MW swimming pool type Pakistan Research reactor-I (PARR-I) for up to 120 hours at a neutron flux of $\sim$1.5x10$^{14}$ cm$^{-2}$ s$^{-1}$. The irradiated material was dissolved in concentrated hydrochloric acid, evaporated and taken in distilled water. 

Preparation of $^{90}$Y-EDTMP
EDTMP was dissolved in distilled water or dilute NaOH. $^{90}$Y chloride solution was added to the EDTMP solution. The pH was adjusted to 8. Radiochemical yields were estimated by paper chromatography. Labeling efficiency of $^{90}$Y-EDTMP was more than 98%.

**POLAND**

*Determination of $^{90}$Sr in $^{90}$YCl$_3$ solution using DGA and Sr-Spec resins (extraction chromatography) and by TLC according to USP*

The evaluation of various extraction chromatography resins indicated that strontium can be more efficiently eluted for the DGA resin than for the Sr-spec resin. Paper chromatography (USP) method provided good resolution of Y and Sr, more results are needed to evaluate results statistically. Comparison of paper chromatography (USP) with paper extraction chromatography was initiated late due to the problems in purchasing KSM-17. The project will be continued in order to determine detection and determination limits of each of proposed methods.

Preliminary investigation on antibody biotinylation resulted in the reproducible method of antibody biotinylation when using human IgG and antitubulin monoclonal antibody fragment (scFvTU-20, mw 30 kDa), obtained from Institute of Molecular Genetics, Prague, Czech Republic). The number of coupled biotin molecules was confirmed using commercially available kit for spectrophotometric assay.

The development of method for preparation of human albumin microspheres as potential radionuclide carriers for diagnostic and therapeutic use resulted in preparation of particles of required size range. The particles were labeled with $^{90}$Y efficiently, however their stability in vitro requires further investigation. Preparation of radiocolloids for radiosynovectomy using radionuclides of various beta energies is in progress in collaboration with University of Lublin, Poland.

**SERBIA**

The main object of the research planned for this project was to optimize the procedures for the $^{90}$Y and $^{188}$Re labelling of different compounds as well as their in vitro and in vivo evaluation. Recent work could be presented in three separated parts:

$^{90}$Y and $^{186/188}$Re complexes for tumor therapy and bone palliation

1) $^{90}$Y complexes of HEDP, MDP and DPD
The object of these studies was to research the possibility for $^{90}$Y-complexion of polyphosphonate ligands MDP, HEDP and DPD. A direct labeling method was optimized by varying ligand concentration and pH in the labeling mixture, as well as the reaction temperature and the reaction time. The experimental results have shown that $^{90}$Y-complexes of different polyphosphonate ligands MDP, DPD and HEDP were obtained in high radiochemical purity and with favorable organ uptake, so they may have potential for use in the palliative treatment of bone metastases.

2) $^{90}$Y complexes of DMSA
The labelling of DMSA with $^{90}$Y was carried out by varying experimental parameters such as ligand concentration, pH, time and temperature of the reaction, in order to maximize the labelling yield. Complete results of radiochemical purity control as well as organ distribution study confirmed that $^{90}$Y-DMSA could be obtained with high radiolabelling yield, with high
radiochemical purity and with satisfactory organ distribution study. Therefore, $^{90}\text{Y}$-DMSA could be also a candidate radiopharmaceuticals for tumour therapy and the palliative treatment of bone metastases.

$^{90}\text{Y}$-particulates

1) $^{90}\text{Y}$-colloids for hepatocellular carcinoma: $^{90}\text{Y}-\text{Sb}_2\text{S}_3$ and $^{90}\text{Y}$-Sn colloid

In these studies we try to explore the factors influencing the labeling yield and particle size distribution of $^{90}\text{Y}$-labeled antimony trisulfide and tin colloid. The results of our research have shown that both $^{90}\text{Y}$-labeled colloids can be prepared in high yields under optimized conditions. Labeling efficiency of $^{90}\text{Y}$-labeled antimony trisulfide and tin colloid was >95 and >88%, respectively. Under well-standardized conditions of the preparation, the reproducibility of the particle size and its distribution is very good, within 7-23 and 85-103 nm for $^{90}\text{Y}$-antimony trisulfide and $^{90}\text{Y}$-tin colloid, respectively.

2) Preparation of Y-90 colloids for radiosynovectomy

Micro hydroxyapatite powders, synthesized and characterized in Laboratory for radioisotopes, Institute “Vinča”. The labelling was carried out by use of $^{90}\text{Y}$ in form of $^{90}\text{YCl}_3$. The presented results has shown that $^{90}\text{Y}$-labelled HAp particles could be prepared in high yield as well as with excellent radiochemical purity. The labelled particulates have shown high in vitro stability at 37°C. The results confirmed the dependence of organ distribution of radiolabelled particles on a way of drug administration. After intravenous application of radiolabelled particles, greater uptake of $^{90}\text{Y}$-HAp and $^{90}\text{Y}$-HEDP-HAp was in liver and then in spleen. The results of organ distribution after intra-articular application of radiolabelled particles in rats confirmed that almost 99.1% of radioactive particles. $^{90}\text{Y}$-HEDP-HAp as well as $^{90}\text{Y}$-HAp, localized in the synovium for at least 96 h, with no detectable activity in the other organs. Biological studies carried out in Wistar rats confirmed complete retention of intra-articular injected radioactivity within the synovial cavity of normal animals for up to 96 h post-injection. Stability of 90-yttrium-HAp complexes increased with introducing diphosphonate HEDP as chelator.

Development of Sr-90/Y-90 generator: electrochemical $^{90}\text{Sr}/^{90}\text{Y}$ - generator and QC of $^{90}\text{Y}$

The objectives of this work were:

- preparation of $^{90}\text{Sr}-^{90}\text{Y}$ generator with useful activity of 3.7 GBq (100 mCi);
- electrochemical separation of $^{90}\text{Y}$;
- development of the methods for determination of other chemical and radionuclide impurity;
- transformation solution of $^{90}\text{Y}$ in appropriate form.

The equipment for electrochemical separation was completed with a potentiostat unit Potentiostat/Galvanostat/ZRA, Series G 750 (Gamry Instruments, inc) together with software license FC 350 (Gamry Instruments, inc). The electrolysis cell is a three electrode system housed in a glass cell fitted with an acrylic cap, made in Serbia. In our first experiments the mixture of $^{90}\text{Sr}$ and $^{90}\text{Y}$ with low activity (only few mCi) was used. So, this work involved setting up the facilities, standardization of preparing protocol and improving existing quality control (QC) procedures in order to supply reliable products to the national nuclear medicine community. These preliminary results are the confirmation that we successfully completed the
equipment for preparing of Sr-90/Y-90 electrochemical generator, established the electrochemical separation technique as well as QC of $^{90}\text{Y}$. In next step, after suppling of some higher quantities of $^{90}\text{Sr}$, we could involve production of $^{90}\text{Sr}/^{90}\text{Y}$ generator in order to supply this product to the national nuclear medicine community in Serbia.

**SYRIA**

The development of yttrium-90 generator and other related radiopharmaceuticals were carried out. $^{90}\text{Sr}$-$^{90}\text{Y}$ generator, was developed by using separation technique based on isolation of $^{90}\text{Y}$ from $^{90}\text{Sr}$ by using Sr – Spec resin packed in three columns. The resulting Y$^{90}$ solution is used for preparation of other therapeutic radiopharmaceuticals.

Up to 100 mCi of Sr-90 was loaded on the top of the first column and Y-90 is eluted with 3M nitric acid solution, where the other two columns were played as further purification barriers. The resulting eluate was evaporated and undergone to further purification processes by passing through cation exchange column for trace element removal. The final solution was concentrated and transformed to chloride form. The yield of yttrium-90 was about 90% where it contained less than $\leq 10^{-6}$ % of strontium-90. The produced yttrium-90 solution was tested for different aspects of quality, and it was showed high quality concerning chemical, radiochemical and radionuclide purities. This high quality was reflected in the high quality of the further prepared radiopharmaceuticals. The latter includes $^{90}\text{Y}$-EDTMP, $^{90}\text{Y}$-FHMA, $^{90}\text{Y}$-DOTA-hR3 and $^{90}\text{Y}$-DOTA-Rituximab.

$^{90}\text{Y}$-EDTMP was prepared by dissolving 150 mg of EDTMP in 25% solution of NH$_4$OH and dilution to 10 ml by water, pH was then adjusted to about 8-8.5. The pH =8-8.5, 1ml of the final solution was labeled with 5 mCi of $^{90}\text{Y}$, and pH was adjusted to 6.5 by using 0.1 M of ammonium chloride. The formulation was left for 15 min before testing. The labeling $^{90}\text{Y}$-FHMA was performed by co-precipitation of yttrium and ferric hydroxides under alkaline conditions, as has been done by earlier workers for other metal ions, in brief, to 1 ml $^{90}\text{Y}$Cl3 (74-185MBq or 25 mCi) in 10 ml vial, 4.0 ml of sodium citrate 0.1 M was added, the previous formed particulates in the room temperature solution was stirred vigorously under sonication then after that it centrifuged, sodium citrate 0.1 M was added with 0.5gr of gelatine in 5ml of saline in boiling water bath and the solution was stirred vigorously under sonication, 1ml of sodium hypochlorite 5% was added. The mixture was centrifuged at 3500 rpm for 5 min the supernatant was removed and counted (almost showed no activity). The percentage yield was calculated as the percent radioactivity associate with the radio labeled particles.

*Antibodies Conjugation and Labeling*

DOTA (S-2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-tetraaceticacid) is coupled to the antibodies (hR3 & Rituximab) for labeling with Yttrium-90, by using published methods. The antibody was kept with DOTA in phosphate buffer pH =7.6-8, at 4°C, while the conjugated antibody was purified by using sphenex G50 column. The resulting conjugated DOTA-antibody was labelled with yttrium -90 in ammonium acetate buffer pH=7.5. The labeling efficiency was as high as 98%. Quality control: All quality tests carried out on the produced radiopharmaceuticals revealed high quality products. The labeling yields exceeded 98% and radiochemical purity appeared to be as high as 99%.
THAILAND

Conditions for development $^{90}$Sr/$^{90}$Y generator base on ion-exchange and extraction chromatography have been optimized using inactive Sr in equivalent to 1 Ci Sr-90 and inactive Y in equivalent to 500 mCi of Y-90 and doping with some tracer Sr-85 and Y-90. The ICP(AES), automatic gamma counter and dose calibrator were used to detect inactive metal, Sr-85 and Y-90, respectively. The extraction paper chromatography (EPC) had been validated as a technique to determine amount of contaminated Sr-90 in the Y-90 product which di (2-ethylhexyl) phosphoric acid was used as ligand to complex Y instead of KSM-17. Liquid scintillation chromatography was selected as a technique to determine amount of Sr-90 and Y-90 after the two nuclides had been separated by EPC. GM counting system was also study for a potential method to determine amount of the contaminated Sr-90. Further study need to be done for small scale generator using both methods, the best and most convenient will be selected for scaling up to at least 500 mCi $^{90}$Sr/$^{90}$Y generator.

UNITED KINGDOM

Re-188-DMSA bisphosphonate conjugates are synthesized but not yet available/tested due to difficulties of chemical characterisation. This will not preclude biological evaluation and further progress and this is under way currently with help of Dr Young Don Hong from Korea (IAEA training fellowship). A series of Re-188 tricarbonyl complexes with bifunctional dipicolylamine-bisphosphonate ligands have been prepared, fully characterised and preliminary biological evaluation complete. The results are promising in that the compounds are homogenous, with well-characterised structures (unlike Re-HEDP) with high serum and in vivo stability (unlike Re-HEDP), higher hydroxyapatite binding and higher uptake and retention in bone than Re-HEDP. One paper has been published and another accepted. The ligands can be made available to other partners working with Re-188 generator with the aim of evaluating synthesis, binding to hydroxyapatite, and detailed in vivo evaluation/dosimetry in animals. Later this year we plan to make dithiocarbamate derivatives available to participants for synthesis of Re-188 nitride complexes. The funding for the project expires spring 2010 so further work beyond this point is uncertain.

VIETNAM

In the course of participating in the IAEA-CRP during the last two years, Vietnam has achieved the goal of setting up a $^{90}$Sr/$^{90}$Y generator system based on Supported Liquid Membrane (SLM) technique and also radiolabeling of the eluted $^{90}$Y with antibody, peptides and albumin. A two stage SLM based $^{90}$Sr-$^{90}$Y generator was set up in-house to generate carrier-free $^{90}$Y at different activity levels viz. 5, 20, 50 mCi. The generator system was operated in sequential mode in which 2-ethylhexyl 2-ethylhexyl phosphonic acid (PC88A) based SLM was used in the first stage for the transport $^{90}$Y in 4.0 M nitric acid from source phase where $^{90}$Sr-$^{90}$Y equilibrium mixture is placed in nitric acid medium at pH to 1-2. In the second stage, octyl (phenyl)-N,N-diisobutylcarbamoylmethyl phosphine oxide (CMPO) based SLM was used for the transport of $^{90}$Y selectively to 1.0 M acetic acid which is the best medium for radiolabeling. The eluted $^{90}$Y from the generator was tested for the presence of any traces of $^{90}$Sr using the Extraction Paper Chromatography (EPC) and was found suitable for radiolabeling. The generator system could be upgraded to 100 mCi level successfully due to an expert mission from India through IAEA. The $^{90}$Y product obtained from the generator system was used for radiolabeling of antibody and peptides viz. Rituximab, DOTATATE and albumin particles under different experimental conditions. A new chromatography system could be developed for analyzing $^{90}$Y labeled albumin in PC and ITLC.
WORK DONE WITHIN THE OBJECTIVES OF THE CRP

Summary of achievements in the development of $^{90}$Sr/$^{90}$Y generators

During the first eighteen months of the CRP, different types of $^{90}$Sr/$^{90}$Y generator systems have been developed/used by the participants for elution of $^{90}$Y.

One of the important achievements of the CRP is the development of $^{90}$Sr/$^{90}$Y electrochemical generators by India. Based on the work carried out on the electrochemical generators in India, a prototype electrochemical generator has been designed and the first such generator has been supplied to Cuba. It is expected that in the next six months, detailed evaluation of the performance of the prototype generator would be carried out by Cuba. There is lot of interest among some participating countries like Syria to buy the prototype electrochemical generator after its successful evaluation. Brazil and Serbia plan to develop an electrochemical generator at their laboratories. For this purpose, Serbia needs the supply of 200 mCi (7.4 GBq) of $^{90}$Sr through IAEA.

Poland and Syria used extraction chromatography generators for elution of high purity $^{90}$Y. Syria has plans of upgrading their generators to 500 mCi (18.5 GBq) levels. During the course of the CRP, Poland provided expert guidance to Serbia on the development of therapeutic radiopharmaceuticals. Syria and Serbia requested the supply of Sr-specific resins through the agency.

The supported liquid membrane (SLM) based generators were used by India and Vietnam for routine elution of $^{90}$Y. India could successfully upgrade their generator system to 100 mCi (3.7 GBq) levels and provided expert guidance to Vietnam (through IAEA) to upgrade their SLM based generator to 100 mCi (3.7 GBq) levels.

Cuba has gained a lot of experience with ion exchange generators. During the first part of the CRP, the generator has been upgraded to 320 mCi (11.84 GBq) and the results have been very good. Cuba would continue using the generator and would compare its performance with the prototype electrochemical generator being installed in their laboratories. Thailand plans to initiate work on the development of extraction chromatography and ion exchange generator systems while Pakistan would try to develop a column chromatography generator in their laboratories. Based on the results obtained, they plan to scale up the best generator.

It was decided that with the choice of many $^{90}$Sr/$^{90}$Y generator systems, each country would decide to use the generator system which would be best suited for their local conditions. The CRP provided opportunity for collaboration among many countries for setting up their $^{90}$Sr/$^{90}$Y generator systems as well as upgradation of their facilities for preparing therapeutic radiopharmaceuticals.

Quality control of $^{90}$Y

An important issue related to the $^{90}$Sr/$^{90}$Y generator is the quality control of the $^{90}$Y product. The most important concern is regarding $^{89}$Sr levels, since the maximum permissible levels for $^{90}$Sr, a bone seeker, is extremely low as the maximum body burden is barely 74 kBq (2 μCi). This task is tricky and difficult, since both $^{90}$Sr and $^{90}$Y are pure beta emitters with overlapping β− spectrum and the conventional techniques are not adequately sensitive for this purpose.
In the first 18 months of this CRP several groups dealt with the measurement of $^{90}$Sr in $^{90}$Y samples using the following methodologies:

- Extraction Paper Chromatography (EPC): India, Vietnam, Serbia
- Extraction Chromatography Resin (Spec, DGA): Poland, Syria
- PC-cation exchange paper Wh P81: Poland
- Ion exchange resin: Cuba, Germany
- Beta counter (measurement of the half-life of $^{90}$Y or allowing the complete decay of $^{90}$Y decay): Brazil, Pakistan, Cuba, Syria
- ICP-OES: Brazil

Some countries showed the interest of using the EPC method but could not get the extractant, KSM-17 or PC88A. Vietnam and India provided the groups (Brazil and Poland) with samples by personal contact.

Work was done aiming the validation of the EPC technology, in particular in India and Thailand, and also the PC-cation exchange paper Wh P81 technology in Poland. Both works used the methodology described at USP to validate their results. More work need to be done, especially in the new EPC technology, and other groups will be involved in the work. For this, a protocol is being provided in this report by India in order to describe an established procedure for the EPC technology.

Syria and Serbia have asked for collaboration in the validation of the EPC methodology.

A comment was made towards the quest for a more readily available extractant agent for the EPC technique. Also, it was agreed to study the role of the presence of EDTA in $^{90}$Sr and $^{90}$Y samples.

Apart from the radionuclidic impurity determination in the product, the other impurities such as metals, organic decomposition products are also important concerns. It is logical to address these concerns also in this CRP. In the first 18 months of this CRP several countries used ICP-OES (Brazil and Poland), ICP-MS techniques (Syria) and ICP-AES (Thailand) to assess the metal content in $^{90}$Y samples. Cuba reported the level of metal impurities but commented the need of better analytical tools to get proper results. No group reported the evaluation of the presence of organic impurities in $^{90}$Y but the need is still present, in particular for ion exchange and SLM generators.

It was decided the need of using the labeling of DOTATATE with high specific activity as a final quality control for $^{90}$Y being eluted from the newly developed generators. A protocol for the labeling and quality control of $^{90}$Y-DOTATATE will be provided.

$^{90}$Y-labelled antibodies: direct labeling vs. pre-targeting

$^{90}$Y-antibodies

Some countries; India, Vietnam, Germany and Syria have optimized the conjugation of the commercially available rituximab with DOTA and DTPA and began the labelling with locally
produced $^{90}$Y. Different specific activities were tested and quality control methods by HPLC have been reported. Determination of the number of chelating groups per antibody molecule has not yet optimized and implemented by all participants.

Germany labelled two other antibodies; anti CD25 and ERIC1 to be used in the treatment of Hodgkin lymphoma and small cell lung cancer, respectively. ERIC1 was labelled with $^{90}$Y and tested in animals bearing neuroblastoma tumour.

The biodistribution showed high accumulation in tumour and reproducible and dose dependent tumour response. In particular, the optimal dose for complete tumour regression has been found to be 2 MBq/animal.

Syria and Cuba have worked with the hR3 antibody and labelled it with locally produced $^{90}$Y. In particular, Cuba has accrued extensive experience in the $^{90}$Y labelling of antibodies, including the purification and quality control steps. In the second part of the CRP, Cuba will test the quality of $^{90}$Y produced from two types of generators in the labelling of hR3.

Poland was working on the optimization of the DOTA conjugation using a locally available antibody.

All the participants in these studies showed the need for the supply of DOTA activated ester by the Agency.

$^{90}$Y-biotin-Pretargeting

This sub-project has two aims: one is to show the superiority of pretargeting over directly labeled antibodies in cancer therapy. The second is to compare the therapeutic efficacy of two biotin-derivatives labelled with $^{90}$Y and $^{188}$Re in pretargeting using the same tumor animal model.

Italy performed the pre-clinical evaluation of a new biotin-DOTA conjugate labeled with $^{90}$Y. Italy will supply biotin-DOTA conjugate to Poland, Vietnam, Cuba, India and Syria to perform their own evaluation using the locally produced $^{90}$Y.

Germany proved the advantages of pretargeting showing that tumor cells accumulated twenty times more radioactivity in a pretargeting three steps approach compared to a control which used a non biotinylated antibody.

Germany and Cuba have the capability of performing in-vivo studies in tumor bearing animals. They wish to explore the potential of a pretargeting approach. To this aim, Italy will provide them with the pretargeting protocol and with all the reagents necessary to perform correctly the studies.

A workshop has been proposed to take place in Cologne next July and all the participants interested in pretargeting were welcomed to join.

Radiolabelled particulates for Radiosynoviorthesis (RSO) or hepatocellular carcinoma (HCC)

The radiopharmaceutical for radiosynoviorthesis (RSO) should meet certain requirements. The radionuclide should be attached to a particle that is sufficiently small to be phagocytised but not so small that it might leak from the joint before being phagocytised (the appropriate
size range is usually considered to be from 2 to 20 μm). The binding between radionuclide and particle should be stable throughout the course of the treatment, which, in turn, is determined by the physical half-life of the radionuclide. The radiolabelled particles should be distributed homogeneously in the intra-articular space without initiation of inflammatory response.

Radiolabelled particulates for treatment of hepatocellular carcinomas are effective when introduced loco-regionally by injection into the tumour site or through a vessel directly going to the cancerous organ. $^{90}$Y labelled non-biodegradable particles such as resin microspheres and glass microspheres are widely used for treatment of hepatocellular carcinomas by injection through the hepatic artery.

The results achieved in the last 18 months can be summarized as follows: Different particulates have been developed to be labelled by $^{90}$Y: $^{90}$Y-citrate-colloid (Brazil, Poland), hydroxy-apatite (Brazil, Serbia); with incorporated $^{90}$Y: HSA-Sn(II)-microspheres (Serbia, Vietnam, Poland), ferric hydroxide (Syria), antimony sulfide (Serbia), chromic-phosphate (Cuba), and HSA-micro-spheres, pre-conditioned with metal-binding ligands (DOTA) (Poland). Many attempts have been made to determine the size of the $^{90}$Y-labelled particles: Laser particle size distribution measurements, microscopy, back-scattering methods, other optical analyses, and fractionated filtration.

To determine the in-vitro stability of labelled colloids, TLC-methods using paper or ITLC-strips with different eluents were applied or precipitation and centrifugation with serum and challenging media.

Biodistribution studies were carried out to confirm the in vivo stability by Brazil, Poland, Vietnam and Serbia. First clinical studies are prepared or already carried out by Brazil and Poland. The following tasks shall be solved in the next 18 months:

To find general conditions of labeling:

- particle size, biodegradable/non-biodegradable
- acceptable and comparable methods to determine particle size
- optimization of particle size
- comparable conditions for the formulation of the injection solution:
  - pH, detergents (gelatine or Tween 80)
  - acceptable and comparable methods for in-vitro and in-vivo stability
  - TLC or others?
  - centralized bio-distribution studies after intra-articular or loco-regionally injection
  - approaching medical partners to exploit the results
  - for this, information about specific activities, radiochemical concentration, particle size and pharmaceutical quality are necessary
Further open questions are e.g. the interaction with co-applied substances such as X-ray-contrast media, glucocorticoids or analgetics which could lead to an increased leakage. A protocol to test such effects will be supplied later.

**188Re-radiopharmaceuticals**

**Pretargeting with 188Re-Biotin**

A number of novel 188Re-biotin conjugates have been prepared by the Italian group following different molecular designs, but all based on the chemical motif of the 188Re-nitrido core. The in vitro stability and inertness of these derivatives towards biotin-degradation enzymes have been established as well as in vitro affinity for avidin. A preliminary experiment aimed at elucidating the *in vivo* uptake by avidin has been carried out in mice after intramuscular deposition of colloidal particles embedded with avidin followed by intravenous injection of 188Re-labeled biotin.

This subtopic can be further developed within this CRP through collaboration between Italy, Germany and Cuba. Specifically, Italy will provide all the reagents for preparing the new 188Re radiocompounds and, then, their biological evaluation will be carried out both in Germany and Cuba, on isolated cells and animal models, using the avidin-biotin pretargeting approach and different types of antibodies. Results will be further compared with similar experiments conducted with biotin derivatives labeled with Lu-177 and Y-90. This comparison will be essential for understanding the role of the nuclear characteristics of a selected radionuclide on the observed biological behavior. It is also envisaged to include this subject in the workshop in Germany proposed for 90Y-biotin research.

Brazil worked on the direct labeling of anti-CD20 with 188Re and will provide Germany with the protocols in order to compare with the pretargeting approach.

**188Re-Bisphosphonates**

A series of mononuclear 188Re compounds incorporating a non-coordinated bisphosphonate moiety has been prepared by the UK group using the 188Re-tricarbonyl metallic fragment as labeling system and a dipicolylamine bisphosphonate derivative as bifunctional ligand. The molecular structure of the resulting mononuclear complex (188Re-DPA-Ale) was fully characterized (see UK country report). Affinity of the pendant bisphosphonate group for hydroxyapatite was tested using the structurally identical 99mTc analogue, and results showed a higher retention of this complex on the inorganic matrix in comparison to the commercial bone seeking agent 99mTc-MDP. A preliminary biological evaluation in normal rats demonstrated that 188Re-DPA-Ale accumulated in bones with highest uptake in joints, thus confirming that this new agent exhibits strong affinity for bone tissue.

The development of this new category of mononuclear 188Re compounds constitutes an important advancement in the design of radiometallic agents for the therapy of bone metastases and the main achievement of this subtopic. It can be further developed within this CRP by pursuing the full characterization of the biological properties of these new radiopharmaceuticals. In particular, the UK group will provide all the procedures and ligands for the preparation and quality control of 188Re-bisphosphonate complexes. A collaboration with the group in Poland may further strengthen this investigation through the activation of a PhD program that may also involve the contribution of a veterinary hospital in Hungary having large facilities for studying different animal models.
The Korean group has devised an alternative approach to the preparation of the Re-188 tricarbonyl complex \([^{188}\text{Re} (\text{CO})_3 (\text{OH}_2)_3]^+\). By this method reduction of \([^{188}\text{ReO}_4]^-\) is accomplished through its interaction with a tetrahydroborate group appended to the surface of an exchange resin, thus allowing easy separation of the final product. Since this metallic precursor is a key intermediate for the synthesis of \(^{188}\text{Re}\) radiopharmaceuticals containing the \([^{188}\text{Re} (\text{CO})_3]^+\) fragment, the new method will be transferred to all groups interested in the preparation of this class of \(^{188}\text{Re}\) complexes. In particular, the UK group will try to apply this procedure for increasing the radiochemical yield of formation of \(^{188}\text{Re}\)-bisphosphonate complexes. Brazil labelled anti-CD20 with \(^{188}\text{Re}\) through a direct route and the tricarbonyl approach developed at PSI and wants to receive the resin from Korea to compare the labeling efficiency and radiochemical purity of the labeled antibody.

**COOPERATION AMONG PARTICIPANTS**

- Pakistan and Thailand will seek collaboration with Cuba regarding the \(^{90}\text{Sr}-^{90}\text{Y}\) ion exchange generators
- Cuba will provide hR3 monoclonal antibody to Brazil, India, Pakistan, Germany, Thailand, Poland and Syria
- Germany will provide ERIC1 and anti CD25 to partners able to perform biological evaluations
- Germany offers MS support to perform tests on antibody affinity after modification and labeling
- Italy (Chinol) will supply biotin DOTA derivative for \(^{90}\text{Y}\) labeling to Cuba, Germany, India, Poland, Syria and Vietnam
- Italy (Duatti) will supply kit formulation for the preparation of \(^{99m}\text{Tc}\) and \(^{188}\text{Re}\) biotin complexes to Cuba, Korea, Serbia and Germany
- Italy (Duatti) will provide the kit formulation for the preparation of the \(^{99m}\text{Tc}\) and \(^{188}\text{Re}\) nitride cores to UK and Korea
- Korea will supply the borohydride exchange resin and protocols to Brazil, Cuba, Vietnam and UK
- UK will provide all the procedures and ligands for the preparation and quality control of \(^{188}\text{Re}\)-bisphosphonate complexes to Poland
- India will provide a protocol for the EPC technique
- India will provide assistance in the validation of the EPC technique to Serbia and Syria
- Serbia and Poland will continue the collaboration on the development of techniques for \(^{90}\text{Y}\) labeling peptides and QC
- Italy (Chinol) will collaborate with Germany and Cuba to perform the animal experiments according to the pretargeting approach

- Serbia will provide samples of Hydroxyapatite to UK

- Cuba will evaluate the $^{90}\text{Sr}-^{90}\text{Y}$ electrochemical generator prototype and will inform MS about the findings

**RECOMMENDATIONS**

- The participants will use a standard protocol for the labeling and quality control of $^{90}\text{Y}$-DOTATATE.

- The work plan of the CRP divided into different components is given in Table 1. Coordinators have been identified for each work component and they will liaise with the other members of the group to monitor the progress of the respective component of the CRP. Overall coordinator of the CRP will liaise with Coordinators of the individual subprograms. *(Attention: Coordinators).*

- The participants will interact with each other on regular intervals. Ms. Renata M. will coordinate a six monthly interaction among the participants.

- The third RCM may be planned during September 2011. The proposed venue is a place where a scientific visit to radiopharmaceutical facilities can be performed, such as Thailand or Germany.

- IAEA may provide antibodies (rituximab) and other reagents (DOTA conjugates) for pre-targeting and antibody labeling which are available commercially.

- IAEA may provide $^{188}\text{W}$ and $^{90}\text{Sr}$ to the requesting MS.

- IAEA may centralize the collection of unpublished medical data and information about the use and application of $^{90}\text{Y}$ and $^{188}\text{Re}$ radiopharmaceuticals from the MS.

**SUMMARY AND CONCLUSIONS**

The important conclusion that emerges from the analysis of results presented at the second research coordination meeting, and that it is worthy to emphasise, is that all research programs outlined during the first RCM in Warsaw have been fully addressed and developed following a clear and rational design. In all projects, important experimental achievements have been obtained and these form a solid and consistent basis where future works can be established.

The search for a suitable and efficient $^{90}\text{Sr}/^{90}\text{Y}$ generator system has led to design and realization of a wealth of different technologies each exploiting a precise physical and chemical approach. The full evaluation of the characteristics and quality of these new generator systems is under active investigation and this work will ultimately allow identification of the most suitable solution. All problems related to the crucial need to obtain a final Y-90 solution of very high chemical and radionuclidic purity have been fully discussed during the meeting as well as the most appropriate techniques for quality control. It is important to note here, that one of these generator systems (electrochemical generator,
Kamadhenu) has already attracted the interest of the industrial world and, thus, its development may receive further support from this participation.

Another important aspect that has received much consideration within the CRP is the use of labeled antibodies for the therapy of different cancers. This subject has been investigated from different perspectives including direct labeling methods and the pre-targeting approach based on the avidin-biotin interaction. In particular, for this latter purpose, various Y-90 and Re-188 biotinylated derivative were prepared and are now available for conducting experimental work on animal tumor models. These results will be compared with data collected in experiments employing Y-90 labeled antibodies radiolabeled through a chelating system directly appended to the antibody’s chain. It is expected that a significant number of biological experiments will flourish with the use of the new radiolabeled biotin derivatives.

Another important step forward in the search for novel bone seeking agents for the therapy of osseous metastases has been brought through the preparation of a series of mononuclear 188Re-complexes tethered to an uncoordinated bisphosphonate moiety. This development could pave the way for the study of 188Re radiopharmaceutical for bone palliation and therapy having a well defined molecular structure, thereby allowing a clear understanding of their underlying biomolecular mechanism of action.

The search for new agents for radiosynovectomy continued in the CRP and different methods have been developed for the stable incorporation of Y-90 into different particle materials (e.g., hydroxyapatite, HSA microspheres and chromium phosphate etc.) and there is potential to do the same with Re-188 also.

All these achievements have led to increase the scientific collaboration between the various participants and this condition will surely constitute a key factor for the successful conclusion of this reaserach project. Finally, it should be noted that the extensive discussion on the work done during the first part of the CRP has allowed precise delineation of scope, methods and experimental plans that will be employed for the further development of the various subprojects.

The workplan for the second half of the CRP has been fully discussed and finalized by all participant groups. Based on the work done during the first part of the project and proposed workplan for the second part, it is expected that the planned objectives of the CRP will be achieved.

**PUBLICATIONS**

**Journals**


   [http://www.rsc.org/publishing/journals/CC/article.asp?doi=B9086](http://www.rsc.org/publishing/journals/CC/article.asp?doi=B9086)


Meetings


5. M. PASQUALI, C. TRAPELLA, R. GUERRINI, A. BOSCHI, L. UCCELLI, E. JANEVIK-IVANOWSKA, A. DUATTI, First application of the IART approach with a new Re-188 labeled biotin derivative. 2009; 52(S1), S248, Poster presentation at 18th International Symposium on Radiopharmaceutical Sciences (ISRS-18) held in Edmonton, Canada, July 13-17, 2009.


### Table 1. WORK PLAN FOR THE SECOND HALF OF THE CRP

<table>
<thead>
<tr>
<th>Scope of the Work</th>
<th>Coordinator</th>
<th>Participating Countries</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. 90Sr/90Y Generator</strong></td>
<td>Meera Venkatesh</td>
<td>Vietnam, India</td>
</tr>
<tr>
<td>1.1 90Sr/90Y SLM Generator</td>
<td>Meera Venkatesh</td>
<td>Vietnam, India</td>
</tr>
<tr>
<td>1.2 90Sr/90Y Electrochemical Generator</td>
<td>Meera Venkatesh/ Ignacio Hernandez</td>
<td>Brazil, India, Serbia, Syria, Cuba</td>
</tr>
<tr>
<td>1.3 90Sr/90Y Extraction Chromatography Generator</td>
<td>Renata Mikolajczak</td>
<td>Syria, Thailand</td>
</tr>
<tr>
<td>1.4 90Sr/90Y Ion-Exchange Chromatography Generator</td>
<td>Ignacio Hernandez</td>
<td>Cuba, Brazil, Pakistan, Thailand</td>
</tr>
<tr>
<td>1.5 Quality Control Techniques: Extraction Paper</td>
<td>Joao Alberto Osso</td>
<td>India, Poland, Cuba, Italy, Brazil, Germany, Serbia, Syria, Thailand, Vietnam</td>
</tr>
<tr>
<td>for Chromatography; Chemical purity (Metallic and organic)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>2. 90Y-Radiopharmaceuticals</strong></td>
<td>Marco Chinol</td>
<td>Italy, India, Vietnam, Germany, Syria, Poland, Cuba</td>
</tr>
<tr>
<td>2.1 90Y- Antibodies</td>
<td>Marco Chinol</td>
<td>Italy, India, Vietnam, Germany, Syria, Poland, Cuba</td>
</tr>
<tr>
<td>2.2 90Y- Biotin- pretargeting</td>
<td>Marco Chinol</td>
<td>India, Germany, Cuba, Poland, Vietnam</td>
</tr>
<tr>
<td>2.3 90Y- particulates/colloids</td>
<td>Klaus Schomäcker</td>
<td>India, Italy, Pakistan, Poland, Serbia, Syria, Vietnam</td>
</tr>
<tr>
<td>For Hepatocellular carcinoma</td>
<td>Klaus Schomäcker</td>
<td>Brazil, Cuba, Germany, Pakistan, Poland, Thailand</td>
</tr>
<tr>
<td>For Radiation Synoviorthesis</td>
<td>Klaus Schomäcker</td>
<td>Brazil, Cuba, Germany, Pakistan, Poland, Thailand</td>
</tr>
<tr>
<td><strong>3. 188Re-Radiopharmaceuticals</strong></td>
<td>Micol Pasquali / Adriano Duatti</td>
<td></td>
</tr>
<tr>
<td>3.1 188Re avidin-biotin pretargeting</td>
<td>Micol Pasquali / Adriano Duatti</td>
<td>Cuba, Poland, Italy, Germany,</td>
</tr>
<tr>
<td>3.2 188Re – DMSA-bis Phosphonates</td>
<td>Rafael Torres Martin de Rosales / Phil Blower</td>
<td>UK, Germany, Serbia, Korea, Pakistan, Poland, Thailand</td>
</tr>
<tr>
<td>3.3 188Re – tricarbonyl labeling strategy</td>
<td>Sang-Hyun Park</td>
<td>Korea, Brazil, Cuba, Germany, uk</td>
</tr>
</tbody>
</table>

Dr. Renata Mikolajczak will continue to be the overall coordinator of the CRP and will liaise with the Coordinators identified for each sub-project.
ANNEX 1: EXTRACTION PAPER CHROMATOGRAPHY (EPC) METHOD FOR ESTIMATION OF 90 Sr CONTENT IN 90 Y CHLORIDE/ACETATE

The following protocol has been designed for the estimation of the radionuclide purity of 90 Y solution.

**Step 1:** Preparation of test solution: A 100 µL of 90 Y acetate ‘test solution’ with a radioactive concentration of 37 MBq/mL (1.0 mCi/mL) is prepared by dilution of the bulk solution with 0.5 M ammonium acetate.

**Step 2:** Preparation of EPC paper: Whatman 1 chromatography paper (12×1 cm) marked into one cm segments is taken and 10 µL of KSM 17 is applied between the 2nd and 3rd segments. The paper is then allowed to dry completely.

**Step 3:** Extraction Paper Chromatography: A 5 µL sample of the ‘test solution’ prepared in Step 1 above is applied on the KSM 17 spot on the EPC paper and allowed to dry completely. The EPC is developed in ascending manner by inserting the paper in a chromatography jar containing 0.9% saline. Care is taken to ensure that the solvent level is below 1 cm mark of the paper. After the solvent has moved to the top, the paper is removed, cut into 1 cm segments and 3 segments of the solvent front inserted in a liquid scintillation vial containing 10 mL of cocktail. The samples are counted for 60 min in a liquid scintillation counter. Please note that the counting needs to be done immediately after completion of the experiment.

Take care while cutting the paper strips not to contaminate the solvent front with the 90 Y activity at the origin.

**Step 4:** Calculation of Results: Using the efficiency of the counter, the activity at the solvent front is calculated. The test solution contained 37 MBq/mL (1.0 mCi/mL) and hence the original activity used in EPC was 1.85×10^5 Bq (5 µCi). The 90 Sr content is calculated from the results. For example, if 6000 counts were obtained in the solvent front for a 60 min counting, this corresponds to 100 cpm, and if the efficiency of the counter is 90%, the activity is 111 dpm or 1.85 Bq. The radionuclidic impurity in this case is 0.001% and the RN purity of the product is 99.999%. The solution thus contains 370 kBq (10 µCi) of 90 Sr in 37 GBq (1 Ci) of 90 Y.
Country report: Brazil

Development of Radiopharmaceuticals Based on $^{188}$Re and $^{90}$Y for Radionuclide Therapy at IPEN-CNEN/SP

18 Months Report, March 2010


Radiopharmacy Center – Institute of Energetic and Nuclear Research – IPE N-CNEN/SP, São Paulo – Brazil

Abstract

The overall objective of this CRP is to develop radiopharmaceuticals for targeted therapy using $^{188}$Re and $^{90}$Y and to study the performance of generators with long lived parent radionuclides as well as to validate the QC control procedures for estimating the purity of generator eluents. The CRP is expected to enhance the capability in production of $^{90}$Y and $^{188}$Re radiopharmaceuticals to meet the increasing demand of therapeutic products for clinical applications, in particular in Brazil. In this period efforts were made towards the assembling of $^{90}$Sr-$^{90}$Y generators, quality control of $^{90}$Y, the labelling of DMSA(V) and anti-CD20 with $^{188}$Re and the labelling of Hydroxiapatite(HA) with $^{90}$Y.

1. $^{90}$Sr/$^{90}$Y generators

1.1 $^{90}$Sr/$^{90}$Y ion exchange chromatography generator

Two generators were prepared based on cation exchange resins, following the work from the previous CRP. The resin employed was Dowex 50W-X8 (100-200 mesh), H$^+$ form and the generators dimensions (Figure 1) were:

- Generator G1: 10 cm high, 1 cm inner diameter
- Generator G2: 10 cm high, 0,5 cm inner diameter

Fig. 1. Ion Exchange generators.
The generators were assembled at a glove box. The loading solution consisted of 3 mCi of $^{90}$SrCl$_2$ (from POLATOM) in 1M HCl. The elutions were performed with 0.003M and 0.03M EDTA solution at pH=4.5. Figure 2 and 3 show the elution profile of and the elution efficiency for both generators, respectively.

![Fig. 2. Elution profile for the ion exchange generators.](image)

![Fig. 3. Elution efficiency for the ion exchange generators.](image)

The mean efficiency values were $(83 \pm 1)\%$ for both generators.

Experiments were performed aiming the EDTA destruction with nitric and perchloric acids. The recovery of $^{90}$Y in chloride form was higher than 95% after 3 destruction processes.

1.2 Quality control

1.2.1 ICP-OES

A methodology of determination of Sr was developed using the ICP-OES methodology. The equipment employed was a Varian Vista – MPX, Varian Inc., EUA. Calibration curves were
performed in decreasing concentrations of Sr certified solutions: 0.2-1 ppm; 0.02-0.1 ppm and 0.002-0.01 ppm. The linear regression analysis of the curves showed good results and values of quantification limits (QL) and detection limits (DL) were calculated. The values found were:

QL: 0.23 ppb  
DL: 0.057 ppb

This means that one can detect the mass of Sr equivalent to a concentration of 0.03 μCi of $^{90}$Sr/mL, calculated solely by the decay of $^{90}$Sr.

1.2.2 Paper chromatography

It was not possible to buy or receive KSM-37 from India, so another complex for Y has been studied, oxime. Some preliminary results showed a potential for its application.

1.2.3 Liquid scintillation counter

A new liquid scintillation counter was acquired and start its operation on March 2010, a Hydex 300SL model (Figure 4).

![Fig. 4. Hydex 300 SL.](image)

This equipment has a three photomultiplier tubes aligned at 120 degrees from each other. These three PMT’s enable triple to double coincidence ratio counting TDCR, allowing a direct way of getting counting efficiency and activity results. Figure 5 shows the spectra obtained from $^{90}$Y eluted from G1 and G2 generators and from the loading solution.
1.3 $^{90}\text{Sr}^{90}\text{Y}$ electrochemical generator

The experiments were performed using a simple electrochemical device (Fig. 6), with two Platinum electrodes acting as cathode and anode. A stabilized DC power source (potentiostat unit) was used with the characteristics: 15 V compliance, 15 $\Omega$ resistance, a maximum current of 150 mA and 60 Hz for impedance (Tectrol, model TC 15-0015, BRAZIL).

The initial experiments were performed using non-irradiated materials (strontium nitrate, Sr(NO$_3$)$_2$, and yttrium oxide, Y$_2$O$_3$) and radioactive tracers (these salts were irradiated at the Nuclear Reactor IEA-R1m, producing $^{88}\text{Y}$ and $^{85}\text{Sr}$ as radiotracers). For the electrolysis using materials non-irradiated, the analysis were performed through the mass difference Sr(NO$_3$)$_2$ and Y$_2$O$_3$ between the electrolysis, using a digital balance (Shimadzu, model AU220D). The gamma activity of $^{85}\text{Sr}$ and $^{88}\text{Y}$ was analyzed using a HPGe detector (Canberra, model 747, USA).

![Fig. 6. Schematic diagram of the electrochemical device used in experiments.](image)

The electrolysis was performed in two stages: the first one aiming the electrodeposition of the desirable element (Y) and the second one, also called recovering stage, aiming the removal of Y from the electrode.
1.3.1 Electrolysis with non-irradiated materials

The electrodeposition stage used ~ 30 mL of a solution containing Sr(NO$_3$)$_2$ or Y$_2$O$_3$ in 1 M HNO$_3$. During the experiments, N$_2$ gas was gently bubbled in the electrolytic solution which was also stirred all the time using a magnetic stirrer. The parameters studied were: time of electrodeposition (between 30-240 minutes), current and voltage applied (60 and 120 mA; 3.0-6.0 V, respectively); pH of the electrolyte solution (1.5-5.0 using 1 M NaOH to adjust) and the concentration of Y. The effect of the presence of N$_2$ was also studied. The Pt electrodes were weighted before and after the electrodeposition in order to evaluate the electrodeposition yield.

The second stage (recovery) was performed removing the electrodes from the electrodeposition experiment, placing them into a clean 0.001-1 M HNO$_3$ solution, reverting the polarity with constant potential and current for a time ranging from 5 to 30 minutes. There was no N$_2$ bubbling, nor stirring in this stage. Again, the electrodes were weighted after the experiments, to evaluate the recovery yield of Y.

The results of the electrodeposition of Y as function of the electrodeposition time, variation of pH and applied current are shown in Figs. 7, 8 and 9, respectively.

![Fig. 7. Electrodeposition of yttrium as a function of electrodeposition time.](image)

![Fig. 8. Electrodeposition of yttrium as a function of electrodeposition time and different values of pH.](image)
The best results were achieved with electrodeposition time between 60 and 90 minutes, pH between 3.5 and 4.0 and current of 60 mA with an average voltage of 5 V. Longer electrodeposition times can lead to the removal of Y from the electrode, whereas short times gave reduced electrodeposition yields. The effect of the best pH conditions also increased the electrodeposition yields for 90 minutes length. The overall best electrodeposition yield was about 60% for Y.

The presence of bubbling gas N₂ during the electrodeposition in the solution for the deposition process was studied. According to the table 2, it’s possible to notice that N₂ bubbling is necessary during the electrodeposition, independent of the quantity of Y₂O₃ in solution, because it releases the gases produced during electrolysis as well as it keeps the solution in dynamic form. The table also shows the advantage of the use of low concentrations of Y₂O₃ in the solution.

<table>
<thead>
<tr>
<th>Electrolysis</th>
<th>Mass of yttrium oxide</th>
<th>Percentage of Y electroplated</th>
</tr>
</thead>
<tbody>
<tr>
<td>With N₂</td>
<td>0.20 g</td>
<td>11%</td>
</tr>
<tr>
<td></td>
<td>0.05 g</td>
<td>27%</td>
</tr>
<tr>
<td>Without N₂</td>
<td>0.20 g</td>
<td>8%</td>
</tr>
<tr>
<td></td>
<td>0.05 g</td>
<td>6%</td>
</tr>
</tbody>
</table>

The results of the experiments of recovery of Y from the electrode are shown in table 3. The best results were achieved using a clean solution of 1 M HNO₃ even in shorter times, when compared to the use of 0.001 M HNO₃.

<table>
<thead>
<tr>
<th>Time of recovering</th>
<th>Percentage of recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 minutes</td>
<td>83%</td>
</tr>
<tr>
<td>30 minutes</td>
<td>79%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time of recovering</th>
<th>Percentage of recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 minutes</td>
<td>97%</td>
</tr>
<tr>
<td>10 minutes</td>
<td>97%</td>
</tr>
</tbody>
</table>

The experiments of electrodeposition of Sr showed that in all conditions studied there was no significant electrodeposition of Sr.

1.3.2 Electrolysis with radiotracers materials
The electrolysis process was performed with a mixture of irradiated Sr(NO₃)₂ and irradiated Y₂O₃. These materials were irradiated at the Nuclear Reactor IEA-R1m (IPEN/CNEN-SP), producing ⁸⁸Y (gamma emitter, t₁/₂=106.64 days) and ⁸⁵Sr (gamma emitter, t₁/₂=64 days).

The solutions were analyzed by γ-spectroscopy before and after the electrodeposition in order to evaluate the electrodeposition yield, using a HPGe detector. The recovering stage was performed during 10 minutes, and the recovery yield was evaluated measuring the solutions before and after the process in a HPGe detector. At the end of an experiment, the electrodes were washed with 3 M HNO₃ and acetone to clean them up.

The experiment was performed with a mixture of ⁸⁵Sr and ⁸⁸Y, using the best conditions of electrodeposition: current of 60 mA, initial pH = 3.5-4.0 with final pH = 2.5 in each electrolysis, bubbling N₂, low concentration of yttrium oxide in the solution and recovery with 1 M HNO₃ with the same pH of the previous solution (Figure 10). It can be observed that in the first 30 minutes of electrolysis, there was no Y electrodeposition. Only after 60 minutes, yttrium was obtained with an electrodeposition yield of 30 % and recovery of 70 % and less than 10 % of ⁸⁵Sr being electroplated together with ⁸⁸Y. There was no significant electrodeposition with a length of 90 minutes.

![Fig. 10. Percentage of electrodeposition of mixture (⁸⁵Sr+⁸⁸Y) radioactive as function of electrodeposition time.](image)

The best conditions for electrodeposition and recovery of Y were established with low contamination with Sr. This method is very suitable to the experimental conditions, so one must be very careful during all the process. These results obtained are useful for the next experiments involving the use of the pair ⁹⁰Sr-⁹⁰Y.

2. **Labelled molecules with ¹⁸⁸Re**

2.1 **Anti-CD 20**

**Reduction of anti-CD20**

Rituximab (10 mg, MabThera/Roche) was reduced by reaction with 2-mercaptoethanol (5 μL, 2-ME/Sigma) at room temperature (RT) for 30 min and the resulting solution was passed through a PD-10 column (Sephadex G-25M, Pharmacia) using phosphate buffered saline (PBS) (pH 7.4) purged with nitrogen as mobile phase and fractions of 1 mL were collected. The concentration of the reduced antibody (Ab) was determined by optical density at 280 nm on a UV/visible spectrophotometer (Hitachi Instruments U-2010). The number of resulting free sulphydryl groups (-SH) was assayed with Ellman’s reagent (Sigma). The average
recovery of the reduced Ab was 98.5 ± 2.6% (average ± SD, n = 4) and the number of -SH groups generated per molecule of Ab was 5.0 ± 1.0 (average ± SD, n = 4).

Radiochemical quality control of $^{188}$Re-RTX

The labeling efficiency was evaluated by instant thin-layer chromatography (ITLC). Both 0.9% NaCl and acetone were used as the mobile phases to separate free perrhenate ($^{188}$ReO$_4^-$). $^{188}$Re-tartrate and $^{188}$ReO$_4^-$ moved with the solvent front ($R_f = 1$) when 0.9% NaCl was used, whereas radiocolloid ($^{188}$ReO$_2$) and $^{188}$Re-RTX remained at the origin ($R_f = 0$). In acetone, $^{188}$Re-RTX, $^{188}$Re-tartrate and $^{188}$ReO$_2$ stayed at the origin and $^{188}$ReO$_4^-$ moved to the solvent front. Human serum albumin (5%)-impregnated ITLC-SG strips were used as the stationary phase and ethanol:ammonia:water (2:1:5 [v/v]) as the mobile phase to separate $^{188}$ReO$_2$ that remained at the origin while the $^{188}$Re-RTX, $^{188}$Re-tartrate and $^{188}$ReO$_4^-$ moved with the solvent front.

Labeling of anti-CD20 and optimization of radiolabeling

The labeling studies were first performed with a liquid formulation of rituximab (RTX) based on the literature that contained: 1 mg of RTX; 82.8 mg of sodium tartrate; 1.67 mg of SnCl$_2$ and 0.25 mg of gentisic acid. Perrhenate eluted from the $^{188}$W/$^{188}$Re generator in 0.9% saline (0.9% NaCl) was added (642.5 MBq). The solution was then incubated for 1 hour at room temperature. The pH was 5.5. The labeling yield was 67.0 ± 0.2%. Because of this low labeling yield, a series of parameters were varied to find a best formulation with higher labeling yield, including Ab mass (0.25, 0.5, 1.0 and 2.5 mg), reducing agent mass (0.25, 0.5, 1.0, 1.67, 3.0, 5.0 and 7.0 mg), tartrate mass (20.7, 41.4, 82.8, 165.6 and 331.2 mg), reaction time (15, 30, 60 and 120 min), $^{188}$Re volume (1 and 2 mL) and activity (3463.2 MBq). The stability at RT, cold temperature (5°C – CT) and dry ice (DI) and at different times (4, 6 and 24 hour) was studied for the optimized formulation. Figures 11, 12, 13 and 14 show the results of the effect of the variation of reducing agent mass, antibody mass, tartrate mass and reaction time on the labeling efficiency of $^{188}$Re-RTX. The volume of 1 mL of $^{188}$Re revealed to be superior to the volume of 2 mL with higher labeling yields and less impurities and when RTX was labeled with high activity of $^{188}$Re the impurities levels of $^{188}$ReO$_2$ and $^{188}$ReO$_4^-$ were more than 25% showing that further studies are necessary to improve the labeling efficiency.

Fig. 11. Radiochemical purity (RP) (%) of $^{188}$Re-RTX with the variation of reducing agent (SnCl$_2$.2H$_2$O)
Fig. 12. RP (%) of $^{188}$Re-RTX with the variation of antibody mass.

Fig. 13. RP (%) of $^{188}$Re-RTX with the variation of tartrate mass.

Fig. 14. RP (%) of $^{188}$Re-RTX with the variation of reaction time.
The stability at room temperature (RT), cold temperature (5°C – CT) and dry ice (DI) and at different times (4, 6 and 24 hour) was studied for the optimized formulation (1.0 mg RTX; 82.8 mg tartrate; 1 mg SnCl₂.2H₂O; 0.25 mg gentisic acid; 1 mL ¹⁸⁸ReO₄⁻, 1 h of reaction) as shown in Figure 15.

Fig. 15. RP (%) of ¹⁸⁸Re-RTX (best formulation) with the passing time and at different temperatures.

Tricarbonyl core technique
A project was developed at PSI, Switzerland in cooperation with Dr. Roger Schibli aiming the labelling of anti-CD20 with ⁹⁹ᵐTc and ¹⁸⁸Re using the tricarbonyl core technique. The study was successful and the results will soon be published.

2.2 DMSA(V)

Radiochemical quality control
The radiochemical purity was evaluated by thin layer chromatography on silica gel (TLC-SG) to determine the labeling efficiency and impurity formation. TLC-SG strips (1.5 x 12 cm) were developed in two different solvent systems. Acetone was used in order to separate ReO₄⁻ (Rf 1) from ¹⁸⁸Re-DMSA(V) and ReO₂ (Rf 0) and 5% glycine was used in order to separate ReO₂ (Rf 0) from ¹⁸⁸Re-DMSA(V) and ReO₄⁻ (Rf 1).

Method I
Initially ¹⁸⁸Re-DMSA(V) was prepared using a commercial kit of DMSA (III) for labeling with ⁹⁹ᵐTc (IPEN-CNEN/SP). The kit contained 1.0 mg de DMSA, 0.44 mg de SnCl₂.2H₂O, 0.70 mg of ascorbic acid and 50 mg de inositol. The labeling was done with 1 mL of ¹⁸⁸ReO₄⁻ (185MBq) and the reaction time was 30 minutes at high temperature (100 °C). The variables studied were: reaction temperature (100 °C and room temperature), reaction time (20 and 30 minutes) and volume of ¹⁸⁸ReO₄⁻ (1.0 and 2.0 mL). Figures 16, 17 and 18 show the results of the effect of the variation of reaction temperature and time and volume on the labeling efficiency of ¹⁸⁸Re-DMSA (V) prepared with a commercial kit of ⁹⁹ᵐTc-DMSA (III). The best results of labeling yield (> 98%) were achieved when it was used 30 minutes of reaction time with heating at 100 °C and 1 mL of ¹⁸⁸ReO₄⁻.
Method II

The second method was prepared in a vial containing 2.5 mg of DMSA (dimercaptosuccinic acid), 1.00 mg of SnCl₂·2H₂O and 30 mg of sodium oxalate, in a total volume of approximately 1 mL. The pH was adjusted to about 5 with 37% HCl. The labeling was done with 1 mL of $^{188}\text{ReO}_4^-$ (185 MBq) and the reaction time was 15 minutes at room temperature. The variables studied were: reducing agent mass (0.2; 0.6; 1.0; 1.5 e 2.0 mg) and mass of DMSA (1.25; 2.5 e 5.0 mg). Figures 19 and 20 show the results of the effect of the variation of the reducing agent and DMSA masses, respectively on the labeling efficiency of $^{188}\text{ReDMSA (V)}$ prepared with method II. The best labeling yield was achieved using 1.0 mg of SnCl₂·2H₂O and 2.5 mg of DMSA but other parameters have to be studied for the optimization of the radiolabeling.
The advantage of this method is that it does not require high temperatures to achieve good labeling yields due to the use of oxalate. This compound complexes with Re in a more appropriate geometry and kinetics promoting a more efficient reduction of $^{188}\text{ReO}_4^-$ when compared with the method I.

3. Routine production of $^{90}\text{Y}$-HA

IPEN has a program of producing colloids for radiation synovectomy and among the products is $^{90}\text{Y}$-HA. The labelling yield is 80% with a radiochemical purity higher than 98%. The mean particle size is 15 $\mu$m. The radioactive concentration is 450-550 MBq/mL. Figure 21 shows the comparison between the application of $^{90}\text{Y}$-citrate (imported) and $^{90}\text{Y}$-HA (home made) in patients.
Fig. 21. Application of $^{90}\text{Y}$-citrate and $^{90}\text{Y}$-HA in patients

Next steps involve the use of home made HA and also use $^{90}\text{Y}$ produced from home made generators for evaluation of their quality.
Country report: Cuba

Local Production of $^{90}\text{Y}$ And $^{188}\text{Re}$ Radionuclides and Development of Radiopharmaceuticals for Therapy

Centro de Isotopos (CENTIS), Cuba

Abmel Xiques, Ignacio Hernández, René Leyva, Marylaine Pérez, Luis Michel Alonso, Minelys Zamora

e-mail: ignacio@centis.edu.cu

Summary

During the first period of this CRP we could test an efficient and reliable generator system based on ion-chromatography to obtain $^{90}\text{Y}$ from its parent radionuclide $^{90}\text{Sr}$. This production scheme for $^{90}\text{Y}$ was outlined in the previous CRP related with the development of generator technologies. Quality parameters such as trace metals that can potentially interfere in the labeling of biomolecules, $^{90}\text{Y}$ recovery, $^{90}\text{Sr}/^{90}\text{Y}$ ratio and radiation dose to bed matrix were evaluated. The results showed that high recovery and radionuclidic purity could be obtained for $^{90}\text{Y}$ during its repeated separation from the $^{90}\text{Sr}$ cow. No replacement or treatment of the cow were necessary and low waste generation and $^{90}\text{Sr}$ losses less that 0.1% after each run were also observed during the present study. A Fab’ fragment was enzimatically produced and purified from the monoclonal antibody h-R3 (Nimotuzumab®). The fragment and the parent antibody were successfully conjugated with DOTA and labeled with $^{90}\text{Y}$. The radioimmunoconjugate thus obtained also exhibited a good 24 h in-vitro stability in an excess of DTPA. A $^{90}\text{Y}$ radiocoloid was prepared in a cromic phosphate particle for radiosynoviorthesis with promising results in animal models. Two alumina based $^{188}\text{W}/^{188}\text{Re}$ generators were prepared and their eluates were used in the labeling of hR3-DOTA conjugates. Quality control and in vivo evaluation in comparison with $^{99}\text{mTc}$-hR3 showed very good results and similar pattern of distribution and pharmacokinetic and will be used in clinical trials for cancer patients.

Introduction

Yttrium-90 and Rhenium-188 are radioisotopes widely used for therapy. Both radioisotopes can be conveniently available from radionuclide generators. Rhenium-188 can be obtained in a ready to use form for instance from an alumina based generator while Yttrium-90 production demands more complicated processing in order to make it suitable for in vivo use. At the same time radiopharmaceuticals based on these two radionuclides are having increasing demand as their efficacy has been proved in applications like cancer treatment, pain palliation and radiation synovectomy.

The development of a sustained national program for these applications requires a reliable production of large amounts of the needed radionuclides and the availability of new molecules to design and produce radiopharmaceuticals with increasing efficacy at the lowest possible cost.

As continuation of the work already performed in the previous IAEA CRP (“Development of Generator Technologies for Therapeutic Radionuclides”) and in correspondence to the research plan outlined in the first coordination meeting of the current CRP held in Poland (July of 2008), our group committed itself to do tasks outlined in Table 1.
Table 1. Working plan as starting point for the first period of the CRP

<table>
<thead>
<tr>
<th>Work related to</th>
<th>Task</th>
</tr>
</thead>
</table>
| **1. **$^{90}$Sr/$^{90}$Y Generator | 1-Evaluation of a $^{90}$Sr/$^{90}$Y Ion-Exchange Chromatography Generator at activities higher than 3.7 GBq  
2-Evaluation of $^{90}$Y Quality Control Techniques:  
- For $^{90}$Sr content :Extraction Paper Chromatography  
- For Chemical purity (Metallic and organic) |
| **2. **$^{90}$Y-Radiopharmaceuticals | 1. Labeling of Mab with $^{90}$Y via:  
- $^{90}$Y- Biotin- pretargeting  
- DOTA-derivatives  
2. Preparation and evaluation of $^{90}$Y- particulates /colloids for radiation synovectomy |
| **3. **$^{188}$Re- Radiopharmaceuticals | $^{188}$Re –pretargeting; via : biotin, haptens  
$^{188}$Re – tricarbonyl labeling strategy |

$^{90}$Sr/$^{90}$Y Generator

Many Ion-exchange chromatographic $^{90}$Sr/$^{90}$Y generators using an organic resin and a chelator/complexor have been described in the literature. Although these systems are simple their main disadvantage relies on the limited radiation stability of the organic exchangers, which limits the activity than can be produced.

In this work, the potentiality of the system composed of the organic cation exchanger Dowex 50Wx8 and the chelating agent EDTA for the production of relatively large quantities of $^{90}$Y was tested. In order to overcome the limitation imposed by the radiation stability of the support, the same principle used by Horwitz and Dietz¹ for the separation of $^{90}$Sr and $^{90}$Y on Sr- and Y-selective resins was employed.

References:

The proposed production scheme is depicted in Fig. 1. The $^{90}$Sr cow was prepared from an 11.8 GBq (320 mCi) $^{90}$Sr solution in 1 M HNO$_3$. After gently evaporating the acid solution the residue was dissolved in 20 mL 6 mM EDTA solution and the pH was adjusted to 4.5. The resultant solution was stored in a 50 mL glass vial. This cow was transferred to the separation system for processing on different $^{90}$Y-ingrowth times. The separation system consisted of three columns (30 cm high x 0.4 cm wide) connected in series. Solutions passed through the columns at 1 mL/min flow rate. Columns were previously washed with 3 FCV of 6 mM EDTA solution pH 4.5. Just before starting the separation, sufficient amount of 0.1 M EDTA solution was added to the $^{90}$Sr cow to make it about 6 mM, in respect to this fresh added solution. Then the pH was adjusted to 4.5-6.0 with diluted NaOH and/or HCl. Followed the $^{90}$Sr/$^{90}$Y-EDTA solution each column was washed with one FCV 6 mM EDTA solution pH 4.5. After discarding an effluent volume equal to the sum of FCV of all columns at the exit of the third column, the solution containing the $^{90}$Y-EDTA complex is collected and treated to remove EDTA and its radiolysis products with a 2 mL mixture (1:1) of concentrated HNO$_3$ and HClO$_4$. The residue containing all the $^{90}$Y is dissolved in 5 mL of 0.1 M HCl. $^{90}$Sr is eluted from the first column with 20 mL of 6 mM EDTA solution pH 11-11.5 back to the cow vial at a flow rate of 0.2 mL/min. The pH of this solution is adjusted to 4-5 by the addition of 350-450 µL of 0.5 M HCl and then it is stored without further treatment until the next separation.

To further improve the quality of $^{90}$Y, it is submitted to a purification process. This procedure was designed to remove as much as possible impurities such as ZrO$_2^{2+}$, Fe$_3^{3+}$, Cu$_2^{2+}$, Zn$_2^{2+}$ that could be present along with $^{90}$Y in the production process. The details of this purification process have been recently reported [2]. Briefly, the $^{90}$Y solution is passed through a 0.4 cm wide x 9 cm high column containing AG50Wx12 resin in H$^+$ form followed by 15 mL of 0.5 M H$_2$SO$_4$ to remove ZrO$_2^{2+}$. Next, 40 mL of 2 M HNO$_3$ are used to remove $^{90}$Sr followed by 25 mL of 2 M HCl to eliminate Fe$_3^{3+}$, Cu$_2^{2+}$, Zn$_2^{2+}$ and other impurities. Lastly 12 mL of 4 M HCl solution are passed through the column to recover the purified $^{90}$Y. The acid is removed by heating and the $^{90}$Y is dissolved in a small volume of 0.01 M HCl solution. The 2 M HNO$_3$

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fraction collected from the column is employed to assess the radionuclidic purity of \(^{90}\)Y by liquid scintillation counting.

The results of the separation after four different ingrowths times are summarized in table 1. In all cases very high (>95%) \(^{90}\)Y recovery was achieved. The radionuclidic purity determination of the \(^{90}\)Y solution indicated that \(^{90}\)Sr D.F. between \(10^6\) and \(10^7\) were regularly attained. The time required for the separation (25-55 min) fluctuated depending on the \(^{90}\)Sr bulk solution volume, which was arbitrarily varied between 20 and 40 mL to test the consistency of the procedure. The \(^{90}\)Sr losses were \(\leq 0.1\%\) being the major contribution the \(^{90}\)Sr activity held in the first column after its elution with the basic pH EDTA solution. Additionally, it can be seen than the combination of the separation and purification procedures provided and overall \(^{90}\)Sr D.F. of \(10^8-10^9\). Similar results were obtained for other runs performed on a weekly base.

**Table 2. Results of the separation of 90Y from an 11.8 GBq 90Sr solution under dynamic conditions.**

<table>
<thead>
<tr>
<th>Batch number</th>
<th>90Y Ingrowth time (days)</th>
<th>90Y recovery (%)</th>
<th>90Sr/90Y after separation</th>
<th>90Sr D.F.</th>
<th>90Sr Loss (%)</th>
<th>90Sr/90Y after purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.8</td>
<td>96.2</td>
<td>5.4x10^{-7}</td>
<td>3.2x10^6</td>
<td>0.07</td>
<td>2.4x10^{-9}</td>
</tr>
<tr>
<td>2</td>
<td>7.2</td>
<td>98.4</td>
<td>1.4 x10^{-7}</td>
<td>8.6x10^6</td>
<td>0.1</td>
<td>1.1x10^{-9}</td>
</tr>
<tr>
<td>3</td>
<td>13.1</td>
<td>96.7</td>
<td>2.2x10^{-7}</td>
<td>4.8 x10^6</td>
<td>0.05</td>
<td>2.1x10^{-9}</td>
</tr>
<tr>
<td>4</td>
<td>19.4</td>
<td>97.1</td>
<td>4.1x10^{-7}</td>
<td>2.5 x10^6</td>
<td>0.09</td>
<td>3.9x10^{-9}</td>
</tr>
</tbody>
</table>

* includes the \(^{90}\)Sr activity held in the separation columns and the one determined in the purification procedure

Values measured after decay of samples

Counting errors are less than 2%.

No replacement or treatment of the cow was necessary and low waste generation was also observed. Although further studies on this system could be needed, the parameters of the separation (time, pH range, flow rate, etc.) and the fact that a low EDTA concentration (0.006 M) was enough to satisfactorily separate both radionuclides point up the potentiality of the procedure for the processing of higher (Ci-quantities) activities.

The chemical purity of \(^{90}\)Y was assessed by subjecting aliquots of \(^{90}\)Y final product after decay to different analytical techniques for the determination of metals like Fe (UV-visible spectrophotometry, Fenantroline), Pb, Zn, Cd (polarography) and Cu (atomic absorption spectrometry). The results displayed in table 2 indicate the high purity attained for \(^{90}\)Y.

**Table 3. Average content found for some metals in purified, decayed \(^{90}\)Y samples for four replicates**

<table>
<thead>
<tr>
<th>Metal</th>
<th>Content (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu</td>
<td>2.4 ± 0.5</td>
</tr>
<tr>
<td>Zn</td>
<td>2.1 ± 0.8</td>
</tr>
<tr>
<td>Cd</td>
<td>&lt; 0.02</td>
</tr>
<tr>
<td>Pb</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>Fe</td>
<td>&lt; 6.25</td>
</tr>
</tbody>
</table>
**90Y-Radiopharmaceuticals**

The monoclonal antibody h-R3 (Nimotuzumab®) developed at the Center of Molecular Immunology (CIM) of Cuba is a humanized Mab that inhibits the high-affinity binding of epithelial growth factor (EGF) receptor. The suitability of using this molecule to treat tumors of epithelial origin over-expressing EGF-R has been already proven through different clinical trials. Some of these trials have been conducted with the DOTA conjugate of this Mab labeled with 188Re. Labeling and stabilities studies of these conjugates with 90Y have also been performed with excellent results [3]. In the present work the potential of Fab’ fragments of Nimotuzumab® (produced enzymatically) to be used for therapy and/or imaging is explored.

**Preparation of Monoclonal Antibody Fab’ fragments**

Nimotuzumab® is commercialized in 10 mL glass vials containing 50 mg of the purified monoclonal antibody, diluted in Phosphate-buffered saline solution (pH=7.0 ± 0.5).

Previously, a desalting step on C26/60 Sephadex G-25M column (Pharmacia, Sweden) coupled to an ÄKTA prime Chromatography System (Amersham Pharmacia Biotech, UK) was preformed for Nimotuzumab® in 0.1 M acetate buffer at pH 4.5. The enzymatic digestion was carried out with molar rate 1:50 pepsin/Mab (w/w). The solution was incubated for 12 h at 37 °C (Memmert, Germany) and then neutralized by addition of 2M Tris base. The cleavage mixture was purified by PD-10 Column packed with rProtein A Sepharose Fast Flow (GE Healthcare, USA), pre-equilibrated with 1.5 M Glycine-NaCl buffer pH 8.9 at linear flow rate of 60 cm/h, coupled to ÄKTA prime Chromatography System (Amersham Pharmacia Biotech, UK). The affinity matrix was regenerated with 0.1 M Citric buffer pH 3.0. The protein A pass was applied to PD-10 Column packed with DEAE Sepharose Fast Flow (GE Healthcare, USA), pre-equilibrated with 0.1 M Tris-HCl buffer pH 8.0 at linear flow rate of 60 cm/h, coupled to the ÄKTA prime Chromatography System. The anion-exchange matrix was regenerated with 3 M NaCl solution. The bivalent fragment obtained F(ab’)2 was reduced to Fab’ treatment with 2-mercaptoethanol, molar rate 1:4000 and then purified by Sephadex G-25M gel filtration.

All steps concerning pepsin cleavage and purification protocol were analytically studied by HPLC and non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The HPLC study was performed on a size-exclusion TSK G3000 pre-packed column coupled to an HPLC Equipment (Shimadzu, Japan). The chromatograms were obtained and processed by UV-RAD software package. The non-reducing SDS-PAGE study was performed by Laemmli procedure on a Mini-PROTEAN® III Cell (Bio-Rad, France) with 7.5% of polyacrylamide. Proteins were stained by Coomassie Colloidal for 120 minutes and the developing procedure was performed with water: acetic acid: methanol (50:10:40) mixture for 24 hours. The molecular weight standard for Lane 0 was High Range Precision Plus™ Protein Unstained (Bio-Rad, France). The purity of the fragment was determinate by Gel Densitometry and molecular weight calculation of unknown pass sample was performed by Minitab 15 software package (EMEA Minitab Ltd., UK).

**Preparation of the Fab’ fragment-DOTA conjugate**

The Fab’ fragment was modified with 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid mono N-hydroxysuccinimide commercial ester (DOTA-NHS-ester) applying a procedure previously established for the conjugation of h-R3 molecule [1].

The conjugation reaction was performed at a DOTA/Fab’ molar ratio of 160:1. The reaction mixture was incubated overnight by gently stirring at 16°C. The immunoconjugate was separated from free DOTA by gel filtration chromatography on disposable PD-10 (Amersham Pharmacia Biotech, Uppsala, Sweden) desalting columns. As a mobile phase, NH₄OAc (0.1 M) pH – 7.0 was used and fractions of 0.5 mL were collected. The Concentration of the immunoconjugate was determined exactly by UV-VIS at 280 nm (Ultrospec 3300 pro, Amersham Pharmacia Biotech).

**Fab’-DOTA conjugate labeling with **⁹⁰Y

A 74MBq (2 mCi) ⁹⁰Y aliquot (8.6 µL) in 0.05 M HCl was added to a plastic vial containing 50 µL of ammonium acetate buffer (2 M, pH 7) followed by 100 µL (0.07 mg) of the Fab’-DOTA-h-R3 conjugate. Additionally 50 µL of ammonium acetate buffer (0.5 M, pH 7) was added. The reaction mixture was left at 42 ºC for 1 h and then 10 µL of 10 mM DTPA solution pH 6 was added to complex the non reacting ⁹⁰Y and left for another 15 min at room temperature. Labeling yield was determined by ITLC-SG with a mixture of ammonium acetate (10 %) and methanol 1:1.

**Fig. 2** shows the results of the pepsin digestion and purification of mAb Nimotuzumab® as examined by SDS–PAGE in the non-reducing conditions. Starting with lane 1 where the mAb Nimotuzumab appears, the subsequent lanes correspond to (2) digest mixture, (3) rProtein A column pass, (4) DEAE Sepharose ion-exchange column pass and (5) final, purified Fab’ fragment. It can be seen that the F(ab’)₂ with molecular weight of 110.49 kDa (Table 3) is formed right after the digestion and disappears completely after the final reduction with 2-mercaptoethanol, appearing a single band of 54.67 kDa near the 45 kDa mass standard corresponding to the Fab’ fragment. In Figure 2 can be also seen a band of calculated molecular weight of 72.44 kDa. This species has not been identified and was separated from the F(ab’)₂ fragment right before the reduction with 2-mercaptoethanol by means of a size exclusion column C16/40 Sepharose CL-4B (Pharmacia, Sweden).
Fig. 2. SDS-PAGE under non-reducing conditions of Nimotuzumab digested by pepsin under pepsin:mAb ratio 1:50 (w:w) at 37°C in 0.1mol/L acetate buffer pH 4.5.

Table 4. Molecular weight calculated by the fitted line in figure 1 of species separated by the SDS-PAGE electrophoresis

<table>
<thead>
<tr>
<th>ID</th>
<th>Assignation</th>
<th>Rf (cm)</th>
<th>*Molecular Weight (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>mAb Nimotuzumab</td>
<td>0.8</td>
<td>153.44</td>
</tr>
<tr>
<td>2</td>
<td>F((ab'))(_2)</td>
<td>1.5</td>
<td>110.49</td>
</tr>
<tr>
<td>3</td>
<td>unknown</td>
<td>2.4</td>
<td>72.44</td>
</tr>
<tr>
<td>4</td>
<td>Fab'</td>
<td>3.0</td>
<td>54.67</td>
</tr>
</tbody>
</table>

* Calculated using the fitted line in figure 1

Fig. 3. Size Exclusion HPLC with TSK G3000 pre-packed column of samples obtained in different stages of the production and purification of the Fab' fragment: a) Nimotuzumab (h-R3) in 0.5 M
Phosphate solution, (b) digest mixture in 0.1 M acetate buffer pH 4.5, (c) rProtein A column pass in 1.5 M glycine buffer containing 1.5 M NaCl pH 8.9, (d) DEAE Sepharose ion-exchange column pass in TRIS buffer 25 mM pH 8.1, (d) final, purified Fab’ fragment in 0.5 M Phosphate solution.

The HPLC follow-up of the different stages of the production and purification of the Fab’ fragment are shown in Fig. 3. It can be seen that the major peaks in every step of the purification (b-d) correspond to the F(ab’)2 fragment which is an indication of the high yield it is formed in the digestion. Finally an almost unique peak is observed corresponding to the Fab’ fragment as corroborated by the SDS-PAGE electrophoresis. Purity of this product was found to be higher than 95% calculated from the peak area in the chromatogram.

The labeling efficiency of the Fab’-DOTA conjugate with ⁹⁰Y was 83.5%. This corresponded to a specific activity of 28.6 mCi/mg. An in vitro stability study performed with the ⁹⁰Y-Fab’-DOTA conjugate (after purification) in an excess of 50 mM DTPA revealed a radiochemical purity above 95% in the first 24 h.

These results indicate the feasibility of producing the Fab’ fragment of the h-R3 mAb (Nimotuzumab) by enzymatic digestion with pepsin and that this fragment can be conveniently conjugated and labeled with ⁹⁰Y exhibiting a good stability in vitro. Further studies are required in order to determine (i) the overall yield of the process and the purity of the fragment, (ii) immunoreactivity of this molecule in every stage -after production, conjugation and labeling- (iii) the number of DOTA groups attached to the molecule after conjugation and its effect on immunoreactivity and (iv) identification of possible application.

**Biodistribution and pharmacokinetic of ⁹⁰Y labeled molecules**

Both radiopharmaceutical preparations, ⁹⁰Y-DOTA-hR3 and ⁹⁰Y-DOTA-Fab’, were administered to healthy mice. Biodistribution at 4, 24 and 48h by organ dissection was performed and pharmacokinetic evaluation by blood sample extraction at different time point.

Biodistribution results shows a typical organ distribution of this kind of molecules in which we can distinguish a bit higher bone accumulation at 4h after administration of ⁹⁰Y-DOTA-Fab’ possibly because a lower in vivo stability. Pharmacokinetic was evaluated by compartmental analysis following an IV bolus two compartment model with first order elimination (KINETICA, Thermo Electron Co.). As expected, both radiopharmaceutical candidates showed quite different behavior because the much lower molecular size of antibody fragments respects the parent molecule. The difference is mainly related to blood clearance and distribution volume, higher in the case of ⁹⁰Y-DOTA-Fab’ in comparison with ⁹⁰Y-DOTA-hR3. A low distribution volume of whole antibody at steady state means a central (blood) compartment distribution, as a contrast its fragment showed an extra vascular distribution revealed by its higher distribution volume.

Results related radiolabeling and biological evaluation will be published soon.

**Cromic (III) phosphate as a matrix of colloidal radiopharmaceuticals**

CrPO₄ obtained by coprecipitation following the general Anghileri procedure as modified by Taylor et al. 2003.

\[ 2\text{CrO}_3 + 2\text{H}_3\text{PO}_4 + 3\text{Na}_2\text{SO}_3 = 2\text{CrPO}_4 \downarrow + 3\text{Na}_2\text{SO}_4 + 3\text{H}_2\text{O} \]

Characterization by means of:
- Sedimentation in different solutions measuring optical density by nephelometry.
- Particle size determination by microscopy and filtration.
- Biodistribution in mice and articular leakage in rabbit knee joint.

| Yield of $^{90}$Y incorporation (%) |  
|---------------------|---------------------|
| **During precipitation** | **After precipitation** |
| 1                     | 99.2                | 99.6                |
| 2                     | 99.4                | 99.3                |
| 3                     | 99.1                | 99.4                |
Particle size evaluation:

![Particle size evaluation graph](image)

Imaging in rabbits:

![Imaging in rabbits](image)

Leakage from knee joint:

![Leakage from knee joint](image)

188Re-Radiopharmaceuticals

Two alumina based 100 mCi $^{188}$W/$^{188}$Re generators were prepared according to protocol described in the previous CRP final report$^4$. The activity was supplied by the IAEA. Part of

$^4$ Xiques A, Torres M, Beckford D, Leyva R, Casanova E, Moreno Y. Development of a reproducible methodology for the production of $^{90}$Y from a $^{90}$Sr/$^{90}$Y chromatographic generator. Technical Reports Series No. 470, THERAPEUTIC RADIONUCLIDE
the $^{188}$Re from both generators was used to support a current clinical trial with $^{188}$Re-hR3 for the treatment of ovarian cancer.

**Radiolabeling approach used for $^{188}$Re labeled molecules**

Radiolabeling was carried out by means of Schwarz method\(^5\). 2-mercaptoethanol (2-ME) was added to monoclonal antibody at 1:2000 molar ratio, reduction took place at room temperature for 30 minutes before purification of reduced antibody by gel filtration on PD-10 Sephadex G25 column. Fractions of 0.5 mL were dispensed on glass vials and kept frozen at -20°C.

As reducing system a solution containing sodium glucoheptonate (162.3 mg/mL, Sigma, EEUU), ascorbic acid (13.3 mg/mL, Merck, UK) and stannous fluoride (1.8 mg/mL, Sigma, EEUU) was prepared in NaCl 0.9%. In order to achieve the higher radiochemical purity, to 0.5 mL of reduced antibody 50 to 600 µL of reducing system was added and allowed to stand at room temperature. Radiochemical purity was checked by paper chromatography at different time points. The best result (more than 98%) was obtained adding 600 µL of reducing system to the monoclonal antibody solution.

**Cysteine challenge**

To evaluate $^{188}$Re-hR3 stability a cysteine challenge at 1:300 molar excess was carried out. Analytical procedure was paper chromatography Whatman 3MM and saline solution as mobile phase. Results obtained show a good stability with similar pattern in comparison with $^{99m}$Tc labeling according with previous work of our group and in agreement\(^6\) or better\(^7\) than other’s authors publications.

**Biodistribution and pharmacokinetic of 188Re labeled molecules**

Biodistribution and pharmacokinetic performed in male Wistar rats (CENPALAB, Havana) show a very similar pattern of the molecule radio labeled with $^{188}$Re and $^{99m}$Tc. In Table 5 compared biodistribution is shown. In Fig. 4 and Table 6 we can observe that pharmacokinetic behavior is very similar, no statistical difference was found, in spite of the minor difference graphically visible.


Table 5. Biodistribution of $^{188}\text{Re-hR3}$ and $^{99m}\text{Tc-hR3}$

<table>
<thead>
<tr>
<th>Sample</th>
<th>$^{99m}\text{Tc-hR3}$</th>
<th>$^{188}\text{Re-hR3}$</th>
<th>T test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0.56 +/-0.14</td>
<td>0.77 +/-0.13</td>
<td>N.S</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.13 +/-0.10</td>
<td>0.13 +/-0.09</td>
<td>N.S</td>
</tr>
<tr>
<td>Kidneys</td>
<td>4.48 +/-0.37</td>
<td>2.86 +/-0.35</td>
<td>(p&lt;0.05)</td>
</tr>
<tr>
<td>Heart</td>
<td>0.00 +/-0.00</td>
<td>0.19 +/-0.05</td>
<td>-</td>
</tr>
<tr>
<td>Stomach*</td>
<td>0.00 +/-0.00</td>
<td>0.77 +/-0.28</td>
<td>-</td>
</tr>
<tr>
<td>Small Intest.*</td>
<td>0.32 +/-0.13</td>
<td>0.74 +/-0.24</td>
<td>-</td>
</tr>
<tr>
<td>Large Intest.*</td>
<td>0.72 +/-0.01</td>
<td>3.16 +/-0.85</td>
<td>(p&lt;0.05)</td>
</tr>
</tbody>
</table>

Fig. 4. Pharmacokinetic behavior of radiolabeled hR3. A: $^{99m}\text{Tc-hR3}$, B: $^{188}\text{Re-hR3}$

Table 6. Calculate pharmacokinetic parameters of radiolabeled antibody.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>U</th>
<th>hR3-$^{99m}\text{Tc}$</th>
<th>hR3-$^{188}\text{Re}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1/2 alpha</td>
<td>h</td>
<td>0.80 h</td>
<td>0.56 h</td>
</tr>
<tr>
<td>T1/2 beta</td>
<td>h</td>
<td>7.14 h</td>
<td>5.33 h</td>
</tr>
<tr>
<td>AUC</td>
<td>%D/mL *h</td>
<td>45.84</td>
<td>39.25</td>
</tr>
<tr>
<td>MRT</td>
<td>h</td>
<td>8.88</td>
<td>7.47</td>
</tr>
<tr>
<td>Co</td>
<td>%D/mL</td>
<td>9.89</td>
<td>6.49</td>
</tr>
<tr>
<td>Ke</td>
<td>1/h</td>
<td>0.22</td>
<td>0.17</td>
</tr>
<tr>
<td>Vc</td>
<td>mL</td>
<td>10.11</td>
<td>13.41</td>
</tr>
<tr>
<td>Total Cl</td>
<td>mL/h</td>
<td>2.18</td>
<td>2.55</td>
</tr>
<tr>
<td>Renal Cl</td>
<td>mL/h</td>
<td>1.61</td>
<td>1.68</td>
</tr>
<tr>
<td>Hepatic Cl</td>
<td>mL/h</td>
<td>0.53</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Conclusions

1. Both generators, $^{188}\text{W/188}\text{Re}$ and $^{90}\text{Sr/90}\text{Y}$, were prepared with excellent performance and reliability.
2. $^{90}$Y radiolabeled molecules, a monoclonal antibody and its fragment Fab’, were obtained with high radiochemical purity and evaluated “in vitro” and “in vivo”.

3. $^{188}$Re radiolabeled monoclonal antibody was obtained with high radiochemical purity and evaluated “in vitro” and “in vivo”.

**Remark:** $^{188}$Re-Nimotuzumab was applied in brain tumor patients following a phase I clinical trial. Other two studies will began, a phase II in brain tumor patients and a phase I in ovarian cancer patients.

**Working plan for the next period**

1. Installation of the $^{90}$Sr/$^{90}$Y electrochemical generator.

2. Testing of the generator with 37 GBq (1 Ci) $^{90}$Sr.

3. Quality control of the produced $^{90}$Y.


5. To continue selection and labeling of different biomolecules of interest with $^{90}$Y and $^{188}$Re.

6. Biological evaluation of labeled molecules by *in vivo* and *in vitro* models.

**Acknowledgments**

We are thankful to IAEA for supporting this work by means of our participation in the CRP. We also thank to Centre of Molecular Immunology (CIM, Havana, Cuba) and Centre of Technological Application and Nuclear Development (CEADEN, Havana, Cuba).
Country report: Germany

Preclinical evaluation of Y-90 labelled Rituximab and ERIC-1, two antibodies for tumor therapy

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Summary

This project focuses on harnessing the great potential of radionuclide therapy, using various different vehicles to transport radionuclides into tumor tissues. A central aim of the project will be to manufacture specific vehicle molecules whose tumor affinity and suitability for radioactive coupling have already been proven through laboratory trials on animals and cell cultures at the Department of Nuclear Medicine, University of Cologne and to label it with Y-90. The vectors to be used to transport radionuclides into tumor tissue for treatment are antibodies against lymphomas and neuroblastomas. The technology applied for coupling Y-90 to various antibodies has been developed to a high level in Cologne and is now ready to be transferred and adapted to GMP (Good Manufacturing Practice) conditions. The antibody against NHL can be acquired commercially and must then be modified for binding to the therapeutically active nuclide Y-90. Similarly, the antibody against neuroblastoma must also be modified to bind to Y-90 but is produced in Cologne. To improve the therapeutic value of antibodies we tried to introduce the pretargeting method.

Tumour pretargeting has shown clear advantages over the direct application of labeled antibodies regarding tumour/background ratios. The pretargeting strategy will be evaluated first on cell cultures and the results will be transferred to in vivo experiments on tumour bearing mice. Briefly, the first component of a three step pretargeting strategy consists of the biotinylated antibody. This includes the protocol for determination of the number of biotin molecules per antibody. By using this technique one can have a stock of biotinylated antibody in lyophilised form ready for further experiments. In the second step, commercially available avidin/streptavidin will be used. Third and final step is the binding of radiolabelled ($^{90}$Y, $^{188}$Re) biotin to the tumour cells through the avidin-antibody-bridge after administration of a clearing agent.

Initial evaluations of the potential radiopharmaceuticals has been carried out by in-vitro experiments on cell lines expressing the corresponding antigen.

Work done so far for three-step pretargeting method can be summarized as follows:

- $^{90}$Y-labelling of Biotin-DOTA
- Coupling of biotinylated Rituximab to CD20-positive Raji-cells
- Successful labelling of cells conjugated with a complex of biotinylated antibody and avidin with $^{90}$Y-DOTA-Biotin
- Surviving of a long-lasting permission procedure for animal experiments file number 8.87-51.04.20.09.339 (25th January 2010, 134 mice)
Furthermore we achieved some new results with I-131 and Y-90 labelled ERIC1 antibodies, directed against NCAM-positive neuroblastomas. The radioiodine labelled anti-NCAM antibody ERIC1 seems to be a promising radiopharmaceutical for treatment of neuroblastoma. The optimal dose is 1-2 MBq/animal. This would correspond to about 600-1200 MBq in humans (for a child of 15 kg).

First experiments with $^{90}$Y-labelled ERIC1 have been started and are very promising. These antibodies showed an unequivocal therapeutic effect, whereas I-131-MIBG showed no influence on the tumour growth on much higher radioactivities applied per animal. We plan for the future:

- Improved dose-finding studies
- Fractionation of radiation dose relised by i.v. application of radiolabelled ERIC1
- Investigation of other radioimmunoconstructs directed against NCAM
- Investigation of other tumour types with NCAM on tumour cells
- Pretargeting

$^{90}$Y-90- or $^{111}$In-labelled Rituximab

Objectives

The antibody against NHL can be acquired commercially. The production of Rituximab coupled to $^{90}$Y first involves a non-radioactive modification of the antibody to allow it to form a chemical binding with $^{90}$Y. This is achieved by the coupling of isothiocyanatobenzyl-DOTA to Rituximab.

The finished DOTA Rituximab solution can subsequently be lyophilised to allow interim storage until labelling with the corresponding radionuclide.

The $[^{111}\text{In}]$-indium labelling for pre-therapeutic dosimetry, the $[^{90}\text{Y}]$-yttrium labelling for therapeutic purposes and the quality control procedures will be carried out under clean-room conditions. The quality control of the radiopharmaceutical is carried out by size exclusion HPLC. The final sterile filtration and bottling takes place under sterile conditions. This work must be carried out under clean-room conditions class A. The ready-to-use solutions should have an activity concentration of c. 185 MBq (5 mCi) $^{111}\text{In}$ for dosimetry and c. 740 MBq (20 mCi) $^{90}\text{Y}$ for therapeutic purposes, each in a volume of 3 ml [3].

First Results

Introduction

The introduction of monoclonal antibodies such as Rituximab, in recent years, has brought about decisive progress in the treatment of aggressive and indolent Non-Hodgkin’s lymphoma. Since then an abundance of further antibodies have been developed for use in oncological disorders and are already employed today in clinical practice. A further tried and tested improvement to the unmodified antibody, has been its coupling to the beta-emitters I-131 and Y-90. The aim here is therefore to establish to what extent the type of labelling
affects the biodistribution of the humanised mouse antibody Rituximab and, in particular, whether In-111 is suitable for use as a surrogate for Y-90.

Methods

Prior to the animal experiments, the affinity of the radioimmuno-constructs to CD20-positive Raji cells were measured in vitro. Labelling with I-131 or In-111 and Y-90 by DOTA was performed by standard methods. The various radioimmuno-conjugates (RIC) were injected intravenously into Burkitt lymphoma-bearing SCID mice. Measurement of the biodistribution was carried out at 24 h, 48 h, 72 h and 96 h p.i. by taking samples of blood, urine, liver, kidney, spleen, gastrointestinal tract (GIT), femur, muscle, thyroid and tumor and measuring the radioactivity with a well-counter. The results were recorded as percentages of the applied dose per gm organ weight (%ID/g).

Results

The dissociation constants obtained for RIC binding to tumour cells ranged from 50-90 nM. The tumour accumulation for Y-90 DOTA Rituximab at 72 h p.i. was significantly higher (2.6 fold) than for In-111-DOTA Rituximab (Y-90: 69.7 % ID/g; In-111: 25.4 % ID/g, 96 h p.i.). Y-90-DOTA Rituximab, in contrast to In-111 analogues, showed a markedly delayed blood clearance, a 2.5-fold higher accumulation in the spleen and a significantly higher accumulation in bone (3-fold). The radioiodide labelled RIC displayed markedly lower accumulation in both tissue and tumor (10-fold in tumor). The tumour/background ratios are only in the case of tumor/muscle promising. We observed very low tumour/blood-ratios. The tumour/background-ratios of Y-90- and In-111-DOTA-Rituximab are not really comparable and were higher in the case of the Y-90 labelled antibody.
Conclusions

In-111 is not a suitable substitute for Y-90 for measurement of the accumulation of antibodies in tumors. Y-86-labelled analogues should be used for reliable pre-therapeutic dose calculations for Y-90 RICs. Y-90 DOTA-Rituximab is a promising candidate for radioimmunotherapy. But: The tumour/background ratios are not sufficient yet and must be improved by using pretargeting methods.

Pretargeting Methods

Tumor pretargeting based on the avidin–biotin system has shown clear advantages over the use of directly labeled antibodies in the treatment of solid tumors. Recently, a new potential application of $^{90}$Y-DOTA-biotin radionuclide therapy in breast cancer has been proposed. The Chinol group, Milan Italy has developed IART® the Intra-operative Avidination for Radionuclide Therapy that relies on the avidin-biotin binding system. In fact, the avidination of the anatomical area of the tumor with avidin, directly injected by the surgeon into and around the tumor bed, provides a target for $^{90}$Y-DOTA-biotin injected intravenously one day later.

The major objective for cancer radioimmunotherapy (RIT) is to enhance the effectiveness of the drug by concentrating it at the tumour site with fewer side toxic effects to normal organs. Tumour targeting was successful with large long-circulating radiolabelled monoclonal antibodies (MoAbs), but high radiation doses to normal organs especially liver, blood and bone marrow soon appeared as a significant problem. Therefore conventional RIT (radioactivity directly attached to MoAb) has achieved success in the treatment of leukemias and lymphomas, in which the tumours are radiosensitive and the tumour cells are relatively accessible.

Tumour pretargeting has shown clear advantages over the direct application of labeled antibodies regarding tumour/background ratios. The pretargeting strategy will be evaluated first on cell cultures and the results will be transferred to in vivo experiments on tumour bearing mice.
Briefly, the first component of a three step pretargeting strategy consists of the biotinylated antibody. This includes the protocol for determination of the number of biotin molecules per antibody. By using this technique one can have a stock of biotinylated antibody in lyophilised form ready for further experiments. In the second step, commercially available avidin/streptavidin will be used. Third and final step is the binding of radiolabelled (\(^{90}\)Y, \(^{188}\)Re) biotin to the tumour cells through the avidin-antibody-bridge after administration of a clearing agent.

Initial evaluations of the potential radiopharmaceuticals have been carried out by in-vitro experiments on cell lines expressing the corresponding antigen. According to the results we can now transform the strategies to a mouse line capable for developing the tumors foreseen for the targeting. The permission for these experiments by the local authorities according to German laws about animal protection we received at the beginning of February 2010.

To prove the advantages of the strategy proposed, the biokinetic data resulting from the pre-targeting approach will be compared with those after administration of directly labelled antibodies in the same animal model. The antibody against NHL can be acquired commercially.

The antibodies for direct use without pre-targeting strategy will coupled to \(^{90}\)Y by non-radioactive modification of the antibody to allow it to form a chemical binding with \(^{90}\)Y. This is achieved by the coupling of isothiocyanatobenzyl-DOTA to the antibodies. The finished DOTA antibody solution can be purified and may be subsequently lyophilised to allow interim storage until labelling with the corresponding radionuclide.

**Biotinylation of antibodies**

We used the following protocol for biotinylation:

- Start with a solution of antibody in sodium bicarbonate buffer 0.1 M pH = 8.5 (O/N dialysis at 2-8 oC)
- Prepare a solution of biotinyl-aminocaproic acid N-hydroxysuccinimide ester in DMSO at the same concentration of the antibody

- Add 0.12 ml of the above biotinylation agent to every ml of antibody (molar ratio biotin: Ab = 10 :1) under continuous and slow stirring

- Keep the solution under slow stirring for 2 h at room temperature

- Centrifugation against PBS (pH = 7.4) at 2-8 ºC (at least 2 changes of 5 liters each)

- Filter through a 0.22 µm Millipore, determine the titer and then make aliquots

- Determine the biotinylation yield (number of biotin per molecule of Ab) by HABA method after enzymatic digestion of Ab (see below)

**Labelling of Biotin with Y-90**

The biotin-DOTA, in saline solution, is labelled with Y-90 at a specific activity of 3.7 MBq/µg.

1.0 M sodium acetate at pH 5.0, with a volume equal to that of the radionuclide chloride solution, is used as buffer. The biotin-DOTA solution is added to the buffer and transferred into the radionuclide supplier vial. The mixture is then mixed and heated at 95 ºC for 30 min.

The radiochemical purity of $^{90}$Y-DOTA-Biotin was tested by TLC using ITLC-SG, Gelman Science, Ann Arbor, MI, USA. An aliquot of the radiolabelling solution mixed with 0.2 mL of an avidin-DTPA solution (0.4 mM Av and 2.5 mM DTPA, final pH= 6.0) served as sample and kept at room temperature for 5 min. 5 µL of the sample were spotted on the paper strip. We used isotonic saline solution as eluent.

The detection was done using TLC Scanner (Raytest, Rita Software) ($R_f = 0$ for labelled DOTA-Biotin, $R_f = 1$ for free radiometal). We receive a Y-90-DOTA-Biotin with very high radiochemical purity. No free radiometal could be detected (Figure 4).

![Fig. 4. TLC-Scan of Y-90-DOTA-Biotin](image)

**Three-step pretargeting: First cell experiments**
The following steps were carried out:

2 \times 10^6 \text{ Raji-cells} were suspended in 0.5 ml medium. The antigen number was verified by binding studies to be 2 \times 10^6. The cells were incubated with 0.1 mg biotinylated Rituximab (0.26 ml PBS), 30 min. After centrifugation, the incubation with 0.06 mg Avidin in 50 µl PBS was carried out for 5 min and it was centrifuged and washed twice again. After that, 30 min incubation with 12 kBq ^{90}\text{Y-DOTA-Biotin} (in 500 µl) was carried out. After centrifugation, washing with PBS, and radioactivity measurement of the cell pellets we could determine the yield of ^{90}\text{Y-Biotin-binding}. Radioactivity measurements were performed in comparison with a standard.

<table>
<thead>
<tr>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial radioactivity</td>
<td>10 344 cps</td>
<td>9 897 cps</td>
<td>10 004 cps</td>
</tr>
<tr>
<td>Cell-bound* radioactivity</td>
<td>8 388</td>
<td>7 944</td>
<td>7 566</td>
</tr>
</tbody>
</table>

*After background subtraction and washing
** Raji cells incubated with non-biotinylated Rituximab

Triplicates and a comparison with cells incubated with non-biotinylated antibody showed the results, demonstrated in Table 1.

Work done so far for three-step pretargeting method can be summarized as follows:

- ^{90}\text{Y-labelling of Biotin-DOTA}
- Coupling of biotinylated Rituximab to CD20-positive Raji-cells
- Successful labelling of cells conjugated with a complex of biotinylated antibody and avidin with ^{90}\text{Y-DOTA-Biotin}
- Surviving of a long-lasting permission procedure for animal experiments file number 8.87-51.04.20.09.339 (25th January 2010, 134 mice)

Anti-neuroblastoma antibodies (\textsuperscript{111}\text{In-} or ^{90}\text{Y-ERIC1})

\textbf{Objectives}

Neuroblastoma is the most frequent solid extracranial childhood tumor and also the most common neoplasm in the first year of life. The most frequent metastatic sites are bone, bone marrow and liver. By a multidisciplinary therapeutic approach, based mainly on poly-chemotherapy, an overall 5 year survival rate of approximately 67% can be reached. Children with certain risk factors, such as amplification of neural myc gene, age at diagnosis >2 years or relapsed disease, have a fundamentally poorer prognosis. New diagnostic and treatment modalities are urgently required to offer these children a better chance of a cure.

Tumor targeting with meta-[^{131}\text{I]}jiodobenzylguanidine (\textsuperscript{131}\text{I-MIBG}) is a well-established method for tumor imaging and for treatment of relapsed disease. \textsuperscript{131}\text{I-MIBG} therapy can achieve an objective tumor response rate of 35%, but in fact its role is palliative.
Unfortunately, side effects, including dose-limiting thrombocytopenia, detract from the clinical usefulness, and about 10% of all tumors investigated showed no MIBG uptake at all and could not be treated.

Therefore, a novel strategy of immunolocalization of human neuroblastoma by targeting the neural cell adhesion molecule (NCAM), which is over-expressed on neuroblastoma. In the present study the $^{[131\text{I}]}$-labelled anti-NCAM antibody ERIC1 was investigated in a human neuroblastoma xenograft SCID mouse model for the first time. The main interest focused on the potential of the radioimmunoconjugate for radioimmunotherapy. Therefore, biodistribution studies were carried out to serve as basis for dose calculations.

![Biodistribution of $^{131\text{I}}$-ERIC1 in IMR5-75 tumor-bearing SCID mice.](image)

**Results**

Measurement of organ-specific radioactivity showed low organ-specific uptake (5.33%ID/g (percent of injected dose per gram of tissue) after 72 h), which continuously decreased over the 96 h investigation period, demonstrating clearance of radioactivity. In contrast, tumors accumulated radioactivity continuously up to a peak of 42.07%ID/g at the 96 h time point (31.07%ID/g at 72 h). This specific uptake could be blocked by application of unlabelled ERIC1 antibodies. Measurement of blood specific radioactivity revealed a characteristic clearance over the first 72 h. With 37 Gy, tumor-specific radioactivity reached therapeutic doses after 96 h [2].

**Conclusions**

Our preliminary experiments in Cologne [2] with a radioactive anti-NCAM antibody ($^{[131\text{I}]}$-ERIC1), where high tumor doses were reached in neuroblastoma-xenograft-bearing SCID mice, will be used as a basis for further development of radio-immunotherapeutic (RIT) methods of neuroblastoma treatment. The neuroblastoma antigen NCAM seems to be an ideal target antigen for this. Biokinetic studies with $^{90\text{Y}}/^{111\text{In}}$-DOTA-ERIC1 are currently carried out.
Therapeutic experiments with antibodies against neuroblastoma

**Objectives**

In our previous studies, the anti-NCAM antibody ERIC1, labelled with I-131, showed a very high specific tumor uptake in neuroblastoma-bearing mice. The aim now is to investigate the therapeutic potential of the radioimmuno-construct labeled with I-131 and Y-90 in animal trials.

**Methods**

As animals SCID-mice without T- und B-lymphocytes, but active NK cell system were used.

3 x 10^7 IMR5-75 cells were implanted subcutaneously. ERIC1 antibodies were labelled with I-131 by chloramines-T method and with Y-90 via DOTA-conjugation. Activities applied were 0.5 - 20 MBq/animal. Analysis was performed by measurement of tumour volume, investigation and investigation of blood (Coulter counter). Furthermore, we compared the therapeutic effect of the radiolabelled antibodies with that of I-131-MIBG.

**Results**

![Graph showing reduction of tumour volume](image)

Fig. 6. Reduction of tumour volume in neublastoma-bearing mice after application of Y-90-DOTA-ERIC1 in different activities, in comparison with a control group

The animals treated with I-131 showed a markedly reduced rate of tumor growth after a dose of 1 MBq/animal. The tumor volume in the mice treated with 1 MBq/animal was 1.5 mm^3 after 16 days, while in the control group it had already increased to 6 mm^3. Above 5 MBq/animal the treated animals died from radiation toxicity. This correlated with a marked loss of leukocytes, erythrocytes and thrombocytes for doses exceeding 5 MBq/animal.

A activity of 1.45 MBq/animal Y-90-DOTA-ERIC1 caused also a tumour remission, but with a mortality of 40 % (figure 6). The I-131-MIBG showed no detectable influence on tumour growth up to activities of 10 MBq/animal.
**Conclusions**

The radioiodine labelled anti-NCAM antibody ERIC1 seems to be a promising radiopharmaceutical for treatment of neuroblastoma. The optimal dose is 1-2 MBq/animal. This would correspond to about 600-1200 MBq in humans (for a child of 15 kg).

First experiments with $^{90}$Y-labelled ERIC1 have been started and are very promising. These antibodies showed an unequivocal therapeutic effect, whereas I-131-MIBG showed no influence on the tumour growth on much higher radioactivities applied per animal. We plan for the future:

- Improved dose-finding studies,
- Fractionation of radiation dose realized by i.v. application of radiolabelled ERIC1
- Investigation of other radioimmuno-constructs directed against NCAM
- Investigation of other tumour types with NCAM on tumour cells
- Pretargeting

The I-131 labelled anti-NCAM antibody ERIC1 is a very promising radioimmuno-construct for the treatment of neuroblastomas. The optimal dose is 1-2 MBq/animal. This would correspond to about 600-1200 MBq in humans (for a child of 15 kg). Therapeutic trials with Y-90 labelled ERIC1 have already begun. In the current investigations, the success of the treatment is being tested for low initial tumor volumes.

**References**


Country report: India

Development of Radiopharmaceuticals Based on $^{188}$Re and $^{90}$Y for Radionuclide Therapy

M. Venkatesh, U. Pandey, P.S. Dhami, R. Chakravarty, M. Kameswaran, S. Subramanian, A. Dash, G. Samuel; Bhabha Atomic Research Centre, Trombay, Mumbai 400 085, India.

1. INTRODUCTION

During the past decade, our group has focused attention on the development of therapeutic radiopharmaceuticals incorporating radioisotopes such as $^{90}$Y, $^{188}$Re etc. As the primary source of the radioisotopes $^{90}$Y and $^{188}$Re are the $^{90}$Sr/$^{90}$Y generators and $^{188}$W/$^{188}$Re generators respectively, the local availability of these generator systems is very important for the successful development of radiopharmaceuticals incorporating these radioisotopes. In this context, $^{90}$Sr/$^{90}$Y generators based on Supported Liquid Membrane (SLM) [1-3] and electrochemical techniques [4] could be designed and deployed in our laboratories for the elution of $^{90}$Y to be used for preparation of $^{90}$Y labeled products. This work formed a part of the IAEA co-ordinated CRP on “Development of Generator Technologies for Therapeutic Radionuclides: $^{90}$Y and $^{188}$Re”.

In this report, work on the development of $^{90}$Y radiopharmaceuticals for treatment of liver cancer and non-Hodgkin’s lymphoma (NHL) is reported. In addition, comparison of the Extraction Paper Chromatography technique (EPC) developed for determination of $^{90}$Sr contamination in $^{90}$Y samples with the US Pharmacopeia recommended method as well as the validation of the EPC technique is presented.

2. DEVELOPMENTS IN THE TWO-STAGE SLM BASED $^{90}$Sr/$^{90}$Y GENERATORS

The SLM based two stage generator could be scaled up to 3.7 GBq (100 mCi) level successfully. Towards such scaling up, $^{90}$Sr was isolated from the high level waste (HLW) arising from spent nuclear fuel to an extent of 18.5 GBq (500 mCi). In the SLM based generator system, the efficient separation of $^{90}$Y from the parent $^{90}$Sr using KSM-17 is highly sensitive to the pH conditions in the feed compartment. Any slight deviation of pH from the ideal conditions may result in poor yield as well as inferior quality of the $^{90}$Y. Hence, in order to reduce the pH sensitivity of the generator system for efficient separation of $^{90}$Y from $^{90}$Sr, an acidic organophosphorous extractant di (2-ethylhexyl) phosphoric acid (D2EHPA) was studied as a potential alternative to KSM-17 in the first stage of the two-stage generator system. Promising results could be obtained in the initial studies with D2EHPA and detailed studies are being carried out to determine the suitability of D2EHPA as the solvent for SLM based separation of $^{90}$Y.

2.1 Validation of the ‘Extraction Paper Chromatography’ (EPC) method for determination of $^{90}$Sr contamination in $^{90}$Y eluted from $^{90}$Sr/$^{90}$Y generator systems

2.1.1 Determination of $^{90}$Sr contamination in $^{90}$Y eluted from $^{90}$Sr/$^{90}$Y Generator Systems by EPC

In the recent past, a novel quality control procedure for detection of $^{90}$Sr in $^{90}$Y eluted from $^{90}$Sr/$^{90}$Y generator systems was developed by our group based on the specific extraction of $^{90}$Y by the chelating agent 2-ethyl hexyl, 2-ethyl hexyl phosphonic acid (KSM-17). The
developed method called Extraction Paper Chromatography (EPC) method [5] makes use of a Whatman 3 mm chromatography paper (12×1 cm) impregnated with 10 μL of KSM-17 at a distance of 2 cm from one end, considered as the origin (Rf=0). The spot is dried and 5 μL of 90Y eluate (37 MBq/mL) from the generator is spotted over the dried spot of KSM-17. The paper is developed in saline. The radioactivity at different regions of the paper is measured using a liquid scintillation counter (LSC) by placing each segment in 10 mL of scintillation cocktail in a scintillation vial. In this system, 90Y chelated to KSM-17 remains at the origin while any trace of 90Sr migrates towards the solvent front.

In order to prove the validity of the EPC technique for 90Sr estimation in 90YCl₃ solutions, it is essential to compare it with an already established and routinely used QC technique for determination of 90Sr in 90YCl₃ solutions used for radiolabeling. The USP monograph for Yttrium-90 Ibritumomab tiuxetan injection describes a chromatography technique using Whatman cellulose phosphate paper for estimating the 90Sr content in 90YCl₃ samples (herein called the reference method) [6]. The EPC technique was compared with the reference method to prove its equivalence to it in terms of the behavior of 90Sr and 90Y in the system.

2.1.2 Comparison of EPC method with reference method

For carrying out the QC procedure as per the reference method, a strontium/yttrium carrier solution containing 0.34 mg of yttrium chloride (YCl₃.6H₂O) and 0.30 mg of strontium chloride (SrCl₂.6H₂O) per mL of 0.1N hydrochloric acid was prepared. About 50 μL of this solution was applied at the origin of a 20×2 cm cellulose phosphate chromatographic strip and allowed to dry. 5 μL of the 90Y chloride solution was applied at the origin and the chromatogram was developed using 3N hydrochloric acid as the developing solvent, until the solvent migrates to the 15 cm mark. It was then allowed to dry. The strip was then cut at the 8 cm mark and the solvent front is placed in a liquid scintillation solvent and counted for presence of 90Sr. The migration behavior of 90Y acetate (obtained from the two stage SLM based 90Sr/90Y generator) and 85+89Sr(NO₃)₂ in EPC was compared to that in the reference method.

Figure 1a shows the migration pattern of 85+89Sr(NO₃)₂ in the EPC method. It can be seen that Sr²⁺ ions are completely migrating towards the solvent front with an Rf = 0.9-1.0. Figure 1b shows the migration pattern of 85+89Sr(NO₃)₂ in the reference method. Here too, Sr²⁺ ions migrate to the solvent front (Rf=0.9-1.0). 85+89Sr tracer was used for these experiments instead of 90Sr since varying amounts of 90Y will be present in 90Sr depending on factors such as the time elapsed after the previous elution, incomplete separation of 90Y and decay of 90Sr. Figure 2a depicts the chromatography pattern of 90Y⁺³ ions in the EPC method. Due to the affinity for KSM-17, 90Y⁺³ ions are retained at the point of application (Rf =0.0). Similar pattern is observed in the reference method also wherein the 90Y⁺³ ions are retained at Rf =0.0 (Figure 2b). Figures 1 and 2 show that Sr²⁺ ions and Y⁺³ ions follow a similar migration pattern in the EPC method and the reference method. This experiment confirms the suitability of the EPC method for estimation of 90Sr contamination in 90Y samples used for preparation of radiopharmaceuticals for therapy.
2.1.3 Recovery of doped $^{85+89}\text{Sr}^{2+}$ as determined by EPC

In order to confirm the efficiency of separation of $^{90}\text{Sr}$ from $^{90}\text{Y}$ using the EPC method, a doping experiment was carried out by doping known counts of $^{85+89}\text{Sr(NO}_3\text{)}_2$ solution in $^{90}\text{Y}$ solution and carrying out the EPC. In this experiment, 5 μL of $^{85+89}\text{Sr(NO}_3\text{)}_2$ solution was mixed with 5 μL of $^{90}\text{Y}$ acetate solution and EPC was carried out. EPC was carried out separately with only 5 μL of $^{85+89}\text{Sr(NO}_3\text{)}_2$ solution for comparison. The solvent front corresponding to $^{85+89}\text{Sr}$ counts was counted in a NaI(Tl) counter and the counts corresponding to $^{85+89}\text{Sr}$ (in the solvent front) were compared to counts of $^{85+89}\text{Sr(NO}_3\text{)}_2$ in order to determine the efficiency of separation by the EPC system. Figure 3a shows the EPC pattern of $^{90}\text{Y}+^{85+89}\text{Sr}$ while figure 3b is the EPC pattern of only $^{85+89}\text{Sr}$. It was observed that almost all the $^{85+89}\text{Sr}$ activity could be recovered in the solvent front of the extraction chromatography paper.
2.1.4 Analysis of long decayed $^{99}$Y samples by EPC technique

$^{90}$YCl$_3$ or $^{90}$Y acetate obtained from $^{90}$Sr/$^{90}$Y generator systems were assigned batch numbers and the samples were allowed to decay for more than 2 months from the date of elution from the generator. After two months, EPC was carried out for all the samples. At the end of 60 days, $^{90}$Y would have decayed by ~22.5 half lives, by which time it is expected that 99.99999% of the $^{90}$Y decays and if any $^{90}$Sr was present, it would still be present. At this juncture, the $^{90}$Sr present would be in secular equilibrium with its in-grown $^{90}$Y and the $^{90}$Sr/$^{90}$Y ratio would be equal to 1. The counts at the origin ($R_f =0$) correspond to $^{90}$Y generated by any $^{90}$Sr contamination which would give an equal count at the solvent front ($R_f=0.9-1.0$). The $^{90}$Sr/$^{90}$Y ratio in this case is expected to be close to 1, if good separation is achieved by the EPC method. This procedure was carried out for several long decayed $^{90}$Y samples in order to confirm the validity of the EPC procedure.

2.1.5 Analysis of serially diluted $^{90}$Y samples

In order to determine the sensitivity of the EPC method at different count rates, $^{90}$Y samples were diluted to different levels and EPC was carried out. Figure 4a shows the EPC pattern of a $^{90}$Y sample (X) and Figure 4b shows the EPC pattern of the same sample diluted 10 folds (X/10). The results from the EPC method gave the dilution ratio as 10.01, indicating its reliable separation efficiency.
2.1.6 Analysis of $^{90}$Y acetate or chloride solutions obtained from Two Stage Supported Liquid Membrane (SLM) based generator and electrochemical generator

Although during the development of EPC method, actual $^{90}$Y eluates from $^{90}$Sr/$^{90}$Y generators were analyzed, as a part of validation, this exercise was carried out again. The developed EPC method was used to determine the $^{90}$Sr content in $^{90}$Y eluates obtained from SLM based two-stage $^{90}$Sr/$^{90}$Y generator and an electrochemical $^{90}$Sr/$^{90}$Y generator. For this purpose, $^{90}$Y was eluted from the SLM based and electrochemical generator systems developed in-house and quality control was carried out using the EPC technique using 5 $\mu$L of 37 MBq/mL sample. The $^{90}$Sr content in various batches of $^{90}$Y eluted from SLM based generators ranged from 12-54 kBq/37 GBq of $^{90}$Y while the $^{90}$Sr levels in the $^{90}$Y eluted from electrochemical generator ranged from 8-14 kBq/37 GBq of $^{90}$Sr, much below the allowed limits.

3. DEVELOPMENT OF $^{131}$I AND $^{90}$Y LABELED RITUXIMAB (ANTI CD20) ANTIBODIES FOR TREATING NON HODGKINS LYMPHOMA

Immunotherapy with monoclonal antibodies (MAb) is an attractive alternative to chemotherapy or radiotherapy in the treatment of B cell lymphoma. This study was carried out with the aim of developing an indigenous radioimmunotherapeutic agent for Non Hodgkin's B cell Lymphoma (NHL). Rituximab is a highly specific human chimeric monoclonal antibody used in the therapy of NHL [7]. Rituximab targets a protein called CD20 which is expressed on the surface of the B cells. Rituximab being a human chimeric antibody does not elicit HAMA response thereby allowing its repeat administration for treatment.

3.1 $^{131}$I labeled Rituximab (anti CD20 antibody)

In this study, 100 $\mu$g of Rituximab procured from a commercial source was radioiodinated with 37 MBq of $^{131}$I using Chloramine T as the oxidizing agent. The radioiodinated antibody was purified by column chromatography on a PD-10 column. In vitro cell binding studies were carried out in Raji and Daudi cell lines which express the CD20 antigen on their surface. Inhibition studies were carried out with cold antibody concentrations ranging from 5 $\mu$g to 100 $\mu$g. Biodistribution and clearance patterns in normal Swiss mice were studied at 3h and 24h post injection with ~370 kBq of radioiodinated antibody.

The radioiodination yields of the anti CD20 antibody was ~ 85-91% (as determined using paper electrophoresis) while the radiochemical purity after purification using PD-10 columns (Fig. 5) was >98%. The $^{131}$I antibody retained its capacity to bind to the cells for a minimum of 10 days. Approximately 15-20% binding was obtained with 105 cells which reduced to ~3% with 50 $\mu$g of cold antibody as shown in figure-6. Biodistribution studies showed significant activity in blood. The clearance was mainly through the gastrointestinal and hepatobiliary system indicating that probably the antibody was degraded in the liver. It is planned to carry out further biodistribution studies in animal models of lymphoma.
3.2 90\textsuperscript{Y} labeled Rituximab

Development of 90\textsuperscript{Y}-radiolabeled monoclonal antibodies (mAbs) usually involves the conjugation of a bifunctional chelating agent (BFCA) to the mAb, followed by labeling of the immunoconjugate with 90\textsuperscript{Y}. In the study para isothiacyanato benzyl DOTA (p-NCS-Bz-DOTA) was used as the BFCA to prepare the antiCD20-DOTA conjugate which was then radiolabeled with 90\textsuperscript{Y}.

3.2.1 Conjugation of antiCD20 with DOTA

The conjugation of anti CD20 with DOTA was carried out at a molar ratio of 1:100 (Ab:DOTA). 2 mL of anti CD20 (10 mg/mL) was concentrated to 1 mL using an AMICON Ultra centrifugal filtration device (MW cut off 10,000). The pH of the solution was adjusted to 9 with bicarbonate buffer. 8.7 mg of p-NCS-Bz-DOTA was added to the pH adjusted Ab solution. The reaction mixture was incubated at room temperature for 1-2 h followed by overnight incubation at 4°C. The excess DOTA was removed by centrifugation in AMICON Ultra centrifugal filter devices at 3500 rpm for 30 min. The process was repeated several times until all the free DOTA was removed. The presence of free unreacted DOTA was monitored using TLC in CH\textsubscript{3}OH:NH\textsubscript{3} (90:10) in which unconjugated p-NCS-Bz-DOTA moves to solvent front. The conjugate was then transferred to a vial using phosphate buffer (pH 7.5) and stored at 4°C.

3.2.2 Radiolabeling of the DOTA-Ab conjugate with 90\textsuperscript{Y}

1 mg of the DOTA-Ab conjugate was taken along with 250 μL of CH\textsubscript{3}COONH\textsubscript{4} buffer, pH 5.5. The pH of 90\textsuperscript{Y} acetate was adjusted to ~5 using 2 N NaOH. ~74 MBq (2 mCi) of the pH adjusted 90\textsuperscript{Y} acetate was added to the conjugate and the mixture was incubated for 3 h at 37°C. The complex was characterized using HPLC. HPLC was carried out on TSK GEL column using 0.05 M PO\textsubscript{4}\textsuperscript{3-} buffer, pH 6.8. Purification of 90\textsuperscript{Y}-DOTA-Ab was carried out on a PD-10 column. The patterns obtained are shown in figure-7. Figure-8 shows the HPLC pattern of pure 90\textsuperscript{Y}-DOTA-anti CD20 Ab. The radiolabeled antibody could be obtained in >98% purity after purification and has a retention time of 10.0 min in the HPLC system. It is planned to carry out further in vitro and in vivo biological evaluation of the product, after further refinement of the DOTA conjugation and radiolabeling procedures.
4. BIOLOGICAL ASSESSMENT OF $^{90}$Y-OXINE IN LIPIODOL AS AN AGENT FOR LIVER CANCER THERAPY

4.1 Preparation of $^{90}$Y oxine

500 µL of 0.4 M ammonium acetate was added to 74-185 MBq (2-5 mCi) of $^{90}$Y acetate. pH of the solution was adjusted to ~6 using 2 N NaOH. To this was added 5 mg of 8-hydroxy quinoline (oxine) dissolved in 200 µL of ethanol. The reaction was carried out at 60°C for one hour. At the end of the reaction, $^{90}$Y-oxine was extracted in dichloromethane. ~70% yield of $^{90}$Y oxine could be obtained under the standardized conditions. The dichloromethane was removed and the radiolabeled complex was suspended in 1 mL of lipiodol for further biological studies. Biological assessment of the $^{90}$Y-oxine complex was carried out using both in vitro and in vivo methodologies.

4.2 In Vitro Assessment

For in vitro assessment, HepG2 human liver carcinoma cell line was employed. This cell line was procured from the National Centre for Cell Science (NCCS), Pune, India, an accredited cell line repository. The cells were cultured in Minimal Essential Medium (MEM) with 10% fetal bovine serum as a growth supplement. They were maintained at 37°C in 5% CO$_2$ atmosphere. Viability of cells was estimated by hemocytometer count with Trypan Blue differential stain. >95% initial viability was the criterion applied when using cells for in vitro assays.

4.2.1 MTT Cell Viability Assay

The effect of exposure to $^{90}$Y-oxine on viability of HepG2 cells was studied using MTT assay. Here, the ability of viable cells to metabolize thiazolyl blue tetrazolium bromide (MTT) to give formazan is used to quantitatively assess cellular viability. Briefly, cells were plated in 96-well plates at 10$^4$ cells/well. Cells were exposed to differing concentrations of $^{90}$Y-oxine (dissolved in ethanol). Total incubation volume was maintained at 200 µL and final ethanol concentration was <0.5%. The cells were exposed to $^{90}$Y-oxine for 24 hours. MTT was freshly dissolved in phosphate buffered saline (PBS) at 5 mg/mL concentration. After 24 hours, 20 µL of the MTT solution was added to each well and mixed gently. This was incubated for 4 hours, after which the supernatant was aspirated completely and the wells rinsed with PBS. Then 200 µL of dimethyl sulfoxide (DMSO) was added to dissolve the
formazan metabolite formed inside the viable cells. This reaction was kept in the dark for 30-45 min and was read on an ELISA reader (560 nm, background corrected at 670 nm). In the MTT assay, >50% loss in cell viability was observed on exposure of $10^4$ cells to a minimum activity of 4.6 MBq of $^{90}\text{Y}$-oxine for 24 h. Further in vitro work was performed at this concentration.

### 4.2.2 LDH release assay

The integrity of the cell membrane was determined by assay of cytoplasmic lactate dehydrogenase (LDH) released into the medium from the HepG2 cells. Here, LDH reduces nicotinamide adenine dinucleotide (NAD) to NADH, which in turn causes the stoichiometric conversion of a tetrazolium dye leading to formation of a colored product. In the assay protocol followed, HepG2 cells were exposed to the labeled preparation (4.6 MBq of $^{90}\text{Y}$-oxine/$10^4$ cells) for ~3 h (controls used cold oxine). The assay for released LDH was performed using LDH-based cytotoxicity assay kit. It was observed that the cells exposed to $^{90}\text{Y}$-oxine showed significant release of cytoplasmic LDH as compared to the controls, indicating loss of cell membrane integrity as a consequence of absorbed dose (Figure-9).

![LDH release assay](image)

### 4.2.3 PCR-based marker assay

Polymerase chain reaction (PCR) assay was performed to assess the expression of various markers associated with apoptotic cell death, on exposure to radiation. The assay was performed on cells exposed for 3 hours to the optimized dose of $^{90}\text{Y}$-oxine. RNA of the cells was isolated using a column purification kit and transcribed to cDNA using reverse transcriptase enzyme reaction. cDNA amplification was performed using PCR and expression of the marker genes was assessed by electrophoresis of amplified DNA. It was observed that the RNA expression of pro-apoptotic marker bax was upregulated on exposure to $^{90}\text{Y}$-oxine as compared to the controls.

### 4.2.4 Caspase-3 assay

This assay was performed to assess the involvement of apoptosis marker protein caspase-3 in the cell death process. Using a colorimetric assay kit, activity of caspase-3 was assayed in Hep G2 cultures exposed to the radiolabeled complex. However, no appreciable difference could be observed between the sample and the unexposed controls. As no protease inhibitor
was used in the assay, it is possible that degradation of caspase-3 in the sample may have occurred and this needs to be investigated further.

4.3 *In vivo* Assessment

*In vivo* assessment was performed in liver cancer model in Wistar Rats. Liver cancer model was raised in the animals by chemical carcinogenesis using diethyl nitrosamine (DEN). The animals were given water ad libitum containing DEN (0.01% w/v). This treatment was carried out for 60 days. After the induction period, the animals were used for *in vivo* experiments. ~37 MBq of $^{90}$Y-oxine in lipiodol (100 μL volume) was administered into the liver via intra-hepatic artery. *In vivo* distribution pattern of the radiolabeled complex at 24h and 96h is given in Figure-10.

![Fig.10. Biodistribution pattern of $^{90}$Y-Oxine in liver cancer model](image)

The *in vivo* biodistribution pattern of $^{90}$Y oxine shows significant disadvantages to its potential application as a liver cancer radiopharmaceutical. At 24h only ~50% of the injected activity is retained in the liver, which reduces to less than 15% at 96 h. It can be seen that a significant proportion of the leached activity appears to localize in the skeletal tissue, where it can give unnecessary dose burden.

**ACKNOWLEDGEMENTS**

The authors express their sincere thanks to IAEA for providing the opportunity to participate in this CRP. The authors are thankful to Director, Radiochemistry & Isotope Group and Director, Nuclear Recycle Group for their support and encouragement. Thanks are also due to the scientists of the groups who have participated in the work reported here.

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Country report: Italy (Chinol)

Pre-clinical evaluation of a new biotin-DOTA conjugate labeled with $^{90}\text{Y}$ for application in pretargeting clinical protocols.

Marco Chinol

INTRODUCTION

In the attempt to improve the therapeutic efficacy of radiolabeled mAbs in cancer radioimmunotherapy, various studies have examined the concept of tumor pretargeting. The so called three-step pretargeting technique, employing the avidin–biotin system, was applied in phase I-II clinical trials showing low toxicity and therapeutic efficacy.

The final step of the pretargeting protocols consists in the systemic injection of radiolabeled biotin. The worldwide recognized “successful association” is between $^{90}\text{Y}$ and the tetraazaamacrocycyle DOTA chelator chemically bound to biotin. Improvements in the structure of the biotin-DOTA conjugate have been reported by our group following a novel approach which simplified the synthetic pattern by reducing the amide group to a methylene group, thus transforming the amide into a secondary amine without affecting the length of the biotin side arm. Preliminary in-vitro experiments, previously published by our group, indicated the potential of the new conjugate.

Based on our previous experience with avidin-based pre-targeting followed $^{90}\text{Y}$-DOTA-biotin in the locoregional treatment of peritoneal carcinomatosis and malignant glioma suggested that similar radionuclide therapy might be worth investigating as a partial replacement of external beam radiotherapy in breast cancer. We have developed IART®, the Intra-operative Avidination for Radionuclide Therapy that relies on the avidin-biotin binding system. In fact, the “avidination” of the anatomical area of the tumor with native avidin, directly injected by the surgeon into and around the tumor bed, provides a target for the radiolabeled biotin intravenously (iv) injected one day later.

In order to optimize the overall strategy, further efforts were needed to optimize the use of the labeled new biotin conjugate and to elucidate its chemical and biological properties. In the first 18 months of this CRP, the pre-clinical evaluation of this new reduced biotinamidohexylamine-DOTA conjugate (ST2210) labeled with $^{90}\text{Y}$ or with $^{177}\text{Lu}$, which is an emerging and promising radionuclide for targeted therapy, was investigated.

EXPERIMENTAL WORK

Effect of pH on radiolabeling. In order to determine the optimal pH for the labeling of ST2210 with $^{90}\text{Y}$ or $^{177}\text{Lu}$, the experiments were performed with pH ranging between 3.6 and 5.6 using 2.0 M sodium acetate buffer. The results, reported in Figure 1, showed that the highest radiochemical purity (RCP) values (near 99%) were obtained in a range of pH 4.4 - 4.8. Out of this range the RCP dropped. In particular, below pH 4.0 or above pH 5.2, RCP was less than 93%. Taking into account these results, the optimal pH (4.4 - 4.8) was adopted in the general procedure using a diluted buffer such as 1.0 M sodium acetate (pH 5.0).
Radiolabeling general procedure. Radiolabeling general procedure has been optimized using ST2210 dissolved in saline at a specific activity of 3.7 MBq/μg. 1.0 M sodium acetate, at pH 5.0, was used to buffer the $^{90}$Y ($^{177}$Lu) chloride solution. The mixture was heated at 95°C for 30 min.

RCP was assayed by Instant Thin Layer Chromatography (ITLC). Briefly, an aliquot (usually 0.05 mL) of the radiolabeling solution was mixed with 0.2 mL of an avidin-DTPA solution (0.4 mM Av and 2.5 mM DTPA, final pH= 6.0) and kept at room temperature for 5 min. Subsequently, 5 μL of the radioactive mixture was spotted on a silica gel ITLC paper strip and then developed in saline solution. In this chromatographic system, Av-radio-biotin complex remains at the origin whereas free radiometal, bound to DTPA, migrates to the solvent front.

Maximum specific activity achievable. To determine the influence of specific activity (SA), expressed as MBq/nmol of ST2210 on RCP value, increasing SAs were tested by labeling biotin either with $^{90}$Y or $^{177}$Lu. The SAs tested ranged from 2.6 to 5.3·10² MBq/nmol.

High SAs were achieved together with high RCPs with both radioisotopes. In particular, RCPs above 99% were obtained with a SA of 5.3 MBq/nmol of $^{90}$Y-ST2210 (Table 1) and with 1.3×102 MBq/nmol of $^{177}$Lu ST2210 (Table 2). A further increase of SA (5.3×102 MBq/nmol for $^{90}$Y-ST2210 and 2.6×102 MBq/nmol for $^{177}$Lu-ST2210) resulted in RCP decrease to 95.0% and 80% respectively.
Table 1. SAs results of $^{90}$Y-ST2210. RCPs > 99% were achieved up to 5.3 MBq/nmol. (n=5)

<table>
<thead>
<tr>
<th>$^{90}$Y (MBq)</th>
<th>biotin (μg)</th>
<th>SA (MBq/nmol)</th>
<th>RCP (%) ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>10</td>
<td>2.6</td>
<td>99.6±0.1</td>
</tr>
<tr>
<td>37</td>
<td>8</td>
<td>3.4</td>
<td>99.6±0.1</td>
</tr>
<tr>
<td>37</td>
<td>5</td>
<td>5.3</td>
<td>99.5±0.3</td>
</tr>
<tr>
<td>37</td>
<td>0.1</td>
<td>$2.6\times10^2$</td>
<td>97.5±0.5</td>
</tr>
<tr>
<td>37</td>
<td>0.05</td>
<td>$5.3\times10^2$</td>
<td>95.0±0.5</td>
</tr>
</tbody>
</table>

Table 2. SAs results of $^{177}$Lu-ST2210. RCPs >99% were achieved up to $1.3\times10^2$ MBq/nmol. (n=5)

<table>
<thead>
<tr>
<th>$^{177}$Lu (MBq)</th>
<th>biotin (μg)</th>
<th>SA (MBq/nmol)</th>
<th>RCP (%) ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>10</td>
<td>2.6</td>
<td>99.6±0.1</td>
</tr>
<tr>
<td>37</td>
<td>5</td>
<td>3.4</td>
<td>99.5±0.1</td>
</tr>
<tr>
<td>37</td>
<td>1</td>
<td>26.4</td>
<td>99.4±0.2</td>
</tr>
<tr>
<td>37</td>
<td>0.5</td>
<td>52.9</td>
<td>99.4±0.3</td>
</tr>
<tr>
<td>37</td>
<td>0.2</td>
<td>$1.3\times10^3$</td>
<td>99.4±0.3</td>
</tr>
<tr>
<td>37</td>
<td>0.1</td>
<td>$2.6\times10^2$</td>
<td>80.0±0.7</td>
</tr>
</tbody>
</table>

Avidin/biotin binding studies. Binding studies were performed by HPLC and by the spectrophotometric method known as HABA assay, based on the use of 4-hydroxazobenzene-2-carboxylic acid. Both methods were aimed at determining the binding stoichiometry of biotin derivative, compared to natural biotin (Vit H), towards avidin at a 1:4 Av/biotin molar ratio which is the saturation ratio for Av/Vit H.

$^{90}$Y- and $^{177}$Lu-ST2210 binding towards Av, determined by HPLC, showed that about 85% of radiolabeled ST2210 was bound to Av at 1:4 molar ratio (Figure 2). The retention time of radiolabeled ST2210-Av complex was 11-12 min whereas radiolabeled ST2210 alone was eluted at 20-22 min. The addition of a large excess of Vit H did not displace the radiolabeled compound from avidin, thus indicating a completely retained affinity of ST2210 for avidin.
As expected, by HABA assay Vit H showed 100% binding to Av at the 1:4 Av/Vit H molar ratio. At the same molar ratio, ST2210 resulted in a binding efficiency towards Av of 85%±1.

Stability studies. Stability studies were performed at 37°C on ST2210 labeled with $^{90}$Y or $^{177}$Lu at a specific activity of 3.7 MBq/μg, either in saline or in a pooled human serum, with and without ascorbic acid (AA) as radical scavenger, at different times of incubation (24, 48, 72, and 96 h).

The studies showed that RCP was higher when AA as radical scavenger was added. Specifically, the RCP was always above 98.0% up to 96 h when the radiolabeled ST2210 was diluted with saline and AA, but decreased to 84.5% at the same time point when dilution was performed with saline only (Figure 3).

Similar AA influence on saline stability emerged also analyzing diluted radiolabeled aliquots (3.7 MBq/0.1 mL). Their stability in saline plus AA was comparable to the results obtained in human serum. In fact $^{90}$Y was tightly bound (98.3%) to ST2210 up to 96 h both in serum and in saline plus AA (Figure 4). Similar behavior was observed for $^{177}$Lu-ST2210.
Fig. 4. Stability of $^{90}$Y-ST2210 aliquots (3.7 MBq/0.1 mL) at 37°C in human serum, saline and saline plus AA. RCPs obtained after serum incubation were higher than those obtained in saline. The results were similar with $^{177}$Lu-ST2210.

FURTHER DEVELOPMENTS

Enhancements of the therapeutic potential of this technique will be evaluated.

New biotin derivatives, carrying two DOTA groups per molecule (BisDOTA), have been synthesized and further efforts will be devoted to elucidate their chemical and biological properties.

The goal will be to label the BisDOTA-biotin at higher specific activity compared to the MonoDOTA analogue. In fact, each biotin can theoretically bind up to two metallic radionuclides. In this way, BisDOTA-biotin radiolabeled at high specific activity could deliver a higher radiation dose to the tumour and then improve the efficacy of targeted radionuclide therapy, provided the high affinity for avidin will be retained.
DEVELOPMENT OF A KIT FORMULATION FOR LABELING BIOTIN-DERIVED LIGANDS WITH THE Re-188 NITRIDO CORE

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1. INTRODUCTION

Within the scope of this CRP, we developed a series of small-molecule biotinylated Re-188 complexes containing the $[^{188}\text{Re}=\text{N}]^{2+}$ core. These complexes have been prepared using different chemical strategies, but all of them incorporate a biologically active biotin group. The main interest for this effort was brought to us after the introduction by Paganelli et al. of a new approach for the treatment of residual malignant cells after surgical removal of a primary breast tumour, and dubbed IART (Intraoperative Avidination for Radionuclide Therapy) [1]. Since now, the IART approach has been applied using $^{90}\text{Y}$ as a therapeutic radionuclide and a DOTA-functionalized biotin ligand for coordination to the radiometal. A major drawback for using $^{90}\text{Y}$ as a therapeutic radionuclide comes from the absence of any radioactive decay, associated with the main $\beta^-$-emission, that may allow imaging of the actual biodistribution of injected radioactivity in any individual patient. Other therapeutic radionuclides, such as $^{177}\text{Lu}$ and $^{188}\text{Re}$, possess an additional $\gamma$-emission that easily allows imaging of target’s uptake, and could be conveniently utilized to monitor the course of therapy. In particular, $^{188}\text{Re}$ emits a $\beta^-$-particle having almost the same energy as that emitted by $^{90}\text{Y}$, but with an associated 155-keV $\gamma$-emission that can be efficiently employed for imaging the biodistribution of the injected radiocompound. Considering also that $^{188}\text{Re}$ is produced through a $^{188}\text{W}/^{188}\text{Re}$ transportable generator system, and that deep similarities between the chemistry of congener rhenium and technetium usually allow $^{99}\text{mTc}$ compounds to be used for a preliminary evaluation of the biodistribution of the analogous $^{188}\text{Re}$ compounds, this makes $^{188}\text{Re}$ as an interesting candidate for application to the IART approach.

The $^{99}\text{mTc}$ and $^{188}\text{Re}$ derivatives developed in our laboratory showed high in vitro affinity for avidin (> 97%), inertness toward in vivo enzymatic degradation, and high uptake in a mouse model of intramuscular in situ deposited avidin followed by intravenous injection of a $^{99}\text{mTc}/^{188}\text{Re}$-biotin conjugate (Fig. 1). Thus, to favor further evaluation of this class of therapeutic agents, we investigated the feasibility of a suitable kit formulation for their high-yield preparation, under strictly sterile and pyrogen-free conditions. As a first approach, we followed a chemical route to the formation of the $[^{188}\text{Re}=\text{N}]^{2+}$ core that we proposed previously and is based on the use of succinic dihydrazide (SDH) as donor of nitrogen donor atoms. Unfortunately, when applied for producing a lyophilized kit, this method revealed to be completely unsatisfactory as final yields were not fully reproducible.

The first studies on the preparation of $^{99}\text{mTc}$-nitrido compounds showed that the simple derivative N-methyl-S-methyl dithiocarbazate [$\text{H}_2\text{N}=\text{N}(\text{CH}_3)\text{C}(-\text{S})\text{SCH}_3 = \text{HDT CZ}$] is a very powerful source of $\text{N}^3$- groups in the reaction with $[^{99}\text{mTcO}_4]$- as only a few micrograms of this reagent are required to afford the $[^{99}\text{mTc}\equiv\text{N}]^{2+}$ core [2–6]. Although it was subsequently found that almost all derivatives containing the basic hydrazine-like motif $>\text{N}$-
N< behave as efficient donors of N\(^3\)− groups, HDTCZ was constantly affording the highest yield of the final ⁹⁹ᵐTc≡N complexes also using micromolar amounts of this reagent in each preparation. The prominent role of HDTCZ as generator of N\(^3\)− groups was sharply confirmed by studies aimed at obtaining the analogous \(^{188}\)Re≡N group from the generator-produced \([^{188}\text{ReO}_4]^-\). The formation of this metallic core in physiological solution is far more difficult than for the ⁹⁹ᵐTc analogue because of the lower standard reduction potential of rhenium complexes. Despite of this, the reaction of \([^{188}\text{ReO}_4]^-\) with DTCZ, in the presence of sodium oxalate and standard amounts (≤ 0.1 mg) of SnCl₂, at room temperature, gives \(^{188}\)Re-nitrido complexes in high yield. On the contrary, other N\(^3\)− donors efficiently employed with \([^{⁹⁹}\text{mTcO}_4]^-\), yield only incomplete conversion of \([^{188}\text{ReO}_4]^-\).

The main driving force that makes HDTCZ an excellent reagent in the formation of the \([\text{M≡N}]^{2+}\) core (M = ⁹⁹ᵐTc and \(^{188}\)Re) can be surely attributed to its combined ability to donate N\(^3\)− groups and, concomitantly, to stabilize the metal center by coordination as a bidentate ligand. Indeed, a number of Tc and Re complexes have been isolated and characterized where HDTCZ is bound to the metal as a monoanionic bidentate ligand (DTCZ) through the neutral sulfur atom of the −C=S moiety and the deprotonated terminal amino group, thus supporting the view that DTCZ coordination would be an important step in the mechanism of formation of the M≡N group. However, the good chelating properties of DTCZ become a disadvantage when exchange reaction by another ligand has to be carried out to obtain the desired final complex. Usually, this can be accomplished by increasing the amount of the exchanging ligand, but this constitutes an important limitation when high specific activities are required.

To overcome this major drawback, we attempted to design of a new dithiocarbazate derivative exhibiting a lower coordinating ability for the metal while preserving its N\(^3\)− donor properties. For this purpose, a sterically encumbering substituent, R, has been appended to the primary amine group of HDTCZ. Accordingly, we report herein on the synthesis of a polyethylene glycol (PEG, average MW, 600 daltons) conjugate of HDTCZ (HO₂C-PEG₆₀₀-DTCZ) and its application to the preparation of a series of asymmetrical ⁹⁹ᵐTc/\(^{188}\)Re nitrido compounds of the type \([\text{M(N)(PNP)(B) }]^{0/+}\) (M = ⁹⁹ᵐTc/\(^{188}\)Re, PNP = diphosphine ligand; B = bidentate ligand) and of 3+1 compounds of the type \([\text{M(N)(L)(P) }]\) (M = ⁹⁹ᵐTc/\(^{188}\)Re; L = tridentate SNS ligand; P = monophosphine).

FIG. 1. Mouse SPECT-CT image of the uptake of \([^{⁹⁹}\text{mTc(N)(Cys-Cys-Biotin)(PTA) }]\) in avidin-embedded colloidal particles deposited through intramuscular injection followed by i.v. injection of Tc-⁹⁹m radioactivity.

2. MATERIALS AND METHODS

All common solvents were reagent grade and were used without further purification. Ligands employed in this study are illustrated in Fig. 2. High performance liquid chromatography (HPLC) was performed on a System Gold Instrument equipped with a programmable solvent Module 126, a scanning detector Module 166, and a radioisotope detector Module 170.
(Beckman Instruments, Fullerton, CA). Where differently not specified, chromatographic analyses were carried out using a reversed-phase C18 Beckman ODS precolumn (25 × 4.6 mm) and a reversed-phase C18 Beckman ODS column (250 × 4.6 mm). Thin-layer chromatography (TLC) was carried out on silica gel plates and reversed phase C18 plates (Merck). TLC chromatograms were analyzed with a Cyclone instrument equipped with a phosphor imaging screen and an OptiQuant image analysis software (Packard, Meridien, CT).

Polyethyleneglycol 600 diacid (HO₂C−PEG₆₀₀−CO₂H), 4-(dimethylamino)pyridine (DMAP), and N,N’-dicyclohexylcarbodiimide (DCC) were purchased from Sigma-Aldrich, Milan, Italy. All moisture-sensitive reactions were performed under a nitrogen atmosphere using oven-dried glassware. Dichloromethane (CH₂Cl₂) and acetonitrile (CH₃CN) were dried over CaH₂ and freshly distilled prior to use.¹H (300 MHz) and 13C (75 MHz) NMR spectra were recorded for acetone-d₆ solutions at room temperature. Assignments were aided by homo- and heteronuclear two-dimensional experiments. ESI MS analyses were performed in positive ion mode with samples dissolved in a mixture of acetonitrile/H₂O 1:1. Crude HO₂C−PEG₆₀₀−DTCZ was purified by preparative reversed-phase HPLC using a Waters Delta Prep 4000 system with a Jupiter column C18 (250 × 30 mm, 300 Å, 15 μm spherical particle size). The column was perfused at a flow rate of 25 mL/min. For the HPLC purification the mobile phase A was H₂O 0.1% TFA while the mobile phase B was CH₃CN 0.1% TFA. A 35 min chromatographic gradient was used to give a linear increase from 0% B to 50% B in 25 min and from 50% B to 100% B in 10 min.

2.1. Synthesis of HO₂C−PEG₆₀₀−DTCZ

To a stirred solution of HDT CZ (544 mg, 4.00 mmol) in anhydrous CH₂Cl₂ (5 mL) polyethyleneglycol 600 diacid (1.20 g, 2.00 mmol), N,N’-dicyclohexylcarbodiimide (DCC, 413 mg, 2.00 mmol), and 4-(dimethylamino)pyridine (DMAP, 24 mg, 0.20 mmol) were added in this order. The formation of a white precipitate was observed after 5 min. To the resulting mixture was then added anhydrous CH₃CN (5 mL). The heterogeneous mixture was stirred at room temperature for an additional 2 days and then concentrated. The residue was suspended in CH₂Cl₂ (100 mL) and then washed with H₂O (2 × 15 mL). The organic phase was dried.
(Na₂SO₄), filtered, and concentrated to give ~1.5 g of a crude material. This residue was then partitioned in Et₂O (100 mL) and H₂O (100 mL). The aqueous phase was extracted with Et₂O (2 × 50 mL) and then concentrated to give of the target HO₂C−PEG₆₀₀−DTCZ derivative (1.10 g, ~75%) slightly contaminated by the starting HO₂C−PEG₆₀₀−DTCZ as judged by ¹H NMR analysis. Purified samples of the target molecule were obtained by preparative HPLC. ¹H NMR (acetone-d₆): δ = 10.2 (bs, ~1 H, CO₂H), 4.21 (s, ~2 H, CH₂CO₂H), 4.19 (s, ~2 H, CH₂CONH), 3.85-3.45 (m, ~47 H, [(CH₂CH₂O)], SCH₃), 2.42 (s, ~3 H, NCH₃). ¹³C NMR (acetone-d₆): δ = 203.1 (CS), 171.8 (CO₂H), 169.0 (CN), 72.0-68.6 (OCH₂), 43.8 (SCH₃), 19.6 (NCH₃). ESI MS (center of a Gaussian distribution): 737.8 (M + H⁺). The synthesis of HO₂C−PEG₆₀₀−DTCZ is outlined in Fig. 3 [7, 8].

FIG. 3. Synthesis of HO₂C−PEG₆₀₀−DTCZ.

2.2. Preparation of ¹⁸⁸Re-complexes

2.2.1. Preparation of [¹⁸⁸Re(N)(PNP)(B)]₀⁺ (B = DBODC, NSH, Cys-Biotin)

Step 1. 5 mg of HO₂C−PEG₆₀₀−DTCZ, 0.2 mg SnCl₂·2H₂O (dissolved in 0.5 mL of 20% v/v aqueous solution of glacial acetic acid) and sodium oxalate (10.0 mg) were placed in a vial purged with nitrogen. Then, 0.5 mL of generator-eluted [¹⁸⁸ReO₄]⁻ (500 MBq) were added, and the resulting mixture was allowed to stand at room temperature for 15 min.

Step 2. 2 mg of PNP, dissolved in 0.5 mL of physiological solution containing 2.0 mg of γ-cyclodextrin and 0.2−0.002 mg of B solubilized in 0.5 mL of physiological solution were simultaneously added to the reaction vial. The resulting solution was heated at 100°C for 30 min. Yield > 93%.

2.2.2. Preparation of [¹⁸⁸Re(N)(L)(P)] (P = PCN, PTA; L = SNS, Cys−Cys−Biotin)

After completing Step 1 as reported above, 1.5 mg of P, dissolved in 0.5 mL of physiological solution (containing 2.0 mg of γ-cyclodextrin when P = PCN) and 0.02 mg of L solubilized in 0.1 mL of physiological solution were simultaneously added to the reaction vial. The resulting solution was heated at 100°C for 30 min. Yield > 90%.
2.3. Purification

To avoid interference of excess of reagents and free ligands on the characterization of the resulting $^{188}\text{Re}$-complexes, the following purification procedures were applied. A C18 SepPak cartridge was activated with 5.0 mL of ethanol and 5.0 mL of deionized water. Successively, the solution containing the complex was diluted with 8.0 mL of deionized water and passed through the cartridge. Approximately 95% of the initial activity was retained on the cartridge. The complex was recovered, after washing with water, by passing 1.5 mL of ethanol.

2.4. Serum Stability

The $^{188}\text{Re}$-complexes (100 µL), purified as described above, were added to a propylene test and incubated at 37°C for 2 h. After incubation, plasma samples were precipitated with acetonitrile and centrifuged (1750g, 5 min). RCP changes in time were monitored by HPLC at 15, 30, 60 and 120 min.

3. RESULTS

3.1. Synthesis and Characterization of HO$_2$C–PEG$_{600}$–DTCZ

The free amine group of the multifunctional HDTCZ was first considered as conjugation site to yield, in a single step, an amidic polyethyleneglycol DTCZ-conjugate by condensation with the commercially available, inexpensive, polydisperse (average MW = 600 Dalton) polyethyleneglycol diacid (HO$_2$C–PEG$_{600}$–DTCZ). The condensation reaction was performed, at room temperature, in a CH$_3$CN/CH$_2$Cl$_2$ mixture (1:1) as solvent, and in the presence of catalytic DMAP and sub-stoichiometric amount (0.5 equiv in respect with carboxylic acid functionality) of the condensation agent DCC. After completion of the reaction (2 days) and removal of the solvent, the optimal purification strategy of the mono-functionalized HO$_2$C–PEG$_{600}$–DTCZ derivative first involved resolubilization of the reaction mixture in CH$_2$Cl$_2$, followed by an aqueous work-up to remove all water-soluble by-products (the solubility of PEGylated DTCZ for CH$_2$Cl$_2$ resulted higher than for H$_2$O). Subsequent evaporation of the organic phase followed by resolubilization in water and extraction of impurities with Et$_2$O allowed to isolate (after concentration of the aqueous phase) the final HO$_2$C–PEG$_{600}$–DTCZ in 75% overall yield and good purity (> 90% by $^1$H NMR analysis). For characterization purposes (NMR and MS analyses) and for application to radiopharmaceutical preparations further purification was also obtained by preparative HPLC.

3.2. Preparation and Characterization of $^{188}\text{Re}$-Complexes

Mixed complexes of the type $[^{188}\text{Re}(\text{N})(\text{PNP})(\text{B})]^{0/+}$ (B = DBODC, NSH, Cys-Biotin; PNP = PNP3, PNP5) and 3+1-substituted complexes of the type $[^{188}\text{Re}(\text{N})(\text{L})(\text{P})]$ (L = Cys-Cys-Biotin; P = PTA, PCN)) were prepared via a similar two-step procedure. In the first step, generator-eluted $[^{188}\text{ReO}_4]$ was mixed with sodium oxalate, HO$_2$C–PEG$_{600}$–DTCZ, and SnCl$_2$ dissolved in aqueous acetic acid and the resulting mixture was allowed to stand at room temperature for 15 minutes. In the second step, the mixture was converted in either the final mixed asymmetrical complex by the simultaneous addition of a B-type ligand and the diphosphine ligand, or in the final 3+1 complex by addition of L-type tridentate ligand and the monophosphine. Heating of the reaction solution at 70 °C for 30 minutes quantitatively converted the intermediate precursor nitride complexes into the final bis-substituted complex $[^{188}\text{Re}(\text{N})(\text{L})_2]$. 
4. DISCUSSION

Aim of this study was the preparation of a new reagent for obtaining Re-188 nitrido radiopharmaceuticals, in high yield and under strictly controlled sterile and pyrogen-free conditions, using a freeze-dried kit formulation. The production of the metal-nitrogen multiple bond, at tracer level, has been successfully achieved with Tc-99m using a variety of reagents all comprising the \( >\text{N}\sim\text{N}< \) moiety. In particular, the compound HDTCZ has been found to be the strongest donor of \( \text{N}_3^- \) groups for Tc-99m. However, succynic dihydrazide (SDH) has been commonly employed to develop new kit formulations for Tc-99m nitrido radiopharmaceuticals (namely, \(^{99m}\text{TeN-NOET}\) and \(^{99m}\text{TeN-DBODC}\)), though it exhibits a weaker donor ability of \( \text{N}_3^- \) groups than HDTCZ. The choice of SDH came about mostly because of its higher solubility in water and lower tendency to coordinate to the metallic center than HDTCZ. Actually, though coordination of HDTCZ to the metal ion greatly improves stabilization of the intermediate metal-nitrido group, its subsequent replacement by other ligands may become difficult.

In attempting to extend this approach to the preparation of analogous Re-188 radiopharmaceuticals, we found that SDH was not so suitable to provide the same yield of formation of the \( [188\text{Re}\equiv\text{N}]^{2+} \) group as observed with Tc-99m under the same mild reaction conditions. In contrast, HDTCZ maintained unaltered its strong \( \text{N}_3^- \) donor ability also towards the reduced Re-188 metallic center, but concomitantly it was able to tightly bind the metal ion, thus forming the neutral bis-substituted symmetrical complex \([\text{Re}(\text{DTCZ})\text{N}]\) (where DTCZ indicates the monodeprotonated form of HDTCZ) or asymmetrical complexes of the type \([\text{Re}(\text{DTCZ})(\text{PNP})]\text{N}\) when reacting in the presence of a diphosphine ligand (PNP). Removal of DTCZ from these intermediate nitrido species is difficult and, usually, can only be accomplished using exceedingly high amounts of exchanging ligand. This constitutes an important limitation in the labeling of bioactive molecules for which a very low amount of bioactive ligands (µg scale) is always necessary to achieve high specific activities.

The polyethyleneglycol conjugate of HDTCZ (HO\(_2\text{C-PEG}_{600}\text{-DTCZ}\)) has been designed to overcome these limitations, in particular by lowering the coordination properties of HDTCZ while keeping unaltered its \( \text{N}_3^- \) donor properties. Accordingly, HDTCZ was modified by appending a polymeric bulky group in a position that could hamper its binding ability. Among the most available polymers, polyethyleneglycol (PEG) is the most successfully used in pharmaceutical science. Because of its unique characteristics, such as absence of immunogenicity and toxicity and high water solubility, PEG is extensively employed for conjugation to organic molecule and small drugs. In this study, HDTCZ PEGylation was performed via amide bond formation by condensation of the primary amine group of HDTCZ with HO\(_2\text{C-PEG}_{600}\text{-DTCZ}\). The new water soluble nitride nitrogen atoms donor HO\(_2\text{C-PEG}_{600}\text{-DTCZ}\) was successfully used to prepare in very high radiochemical purity both asymmetrical mixed complexes of the type \([\text{M}(\text{PNP})(\text{B})]\text{N}^{0/+} \) (B = DBODC, NSH, CysNAc and HDTCZ), and 3+1 compounds of the type \([\text{M}(\text{L})(\text{P})]\text{N} \) (L = Cys-Cys-Biotin; P = PTA, PCN) adding only micrograms of the required ligands (L and B). In turn, HO\(_2\text{C-PEG}_{600}\text{-DTCZ}\) has been employed to develop a two-vial kit formulation for the preparation of the new Re-188 biotin complexes. The stability and efficiency of this new freeze-dried kit for the preparation of the \(^{188}\text{Re}\)-nitrido core is currently under evaluation.

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Country report: Rep. of Korea

188Re-TRICABONYL LABELING STRATEGY: Preparation of 188Re(I) Tricarbonyl Precursor [188Re(OH2)3(CO)3]+ for the Labeling of Biomolecules

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INTRODUCTION

The 99mTc(I) and 188Re(I) tricarbonyl precursors [M(OH2)3(CO)3]+ have been shown to be excellent starting materials for the synthesis of 99mTc(I) and 188Re(I) tricarbonyl complexes as well as radiolabeling of target-specific biomolecules.1-8 Recently, a user-friendly kit formulation (IsoLinkTM) was developed using potassium boranocarbonate, K2[BH3CO2], for preparation of the 99mTc precursor complex. This solid reagent serves both as a source of carbon monoxide and a reducing agent for technetium. It was also used by Schibli and co-workers for the preparation of the corresponding 188Re precursor complex (“Schibli’s kit”).9 This approach involved the reduction of [188Re]perrhenate eluate (1 ml) in neutral solution with 3 mg K2[BH3CO2] and 5 mg BH3 ≅ NH3 by incubation at 60 °C for 15 min. The amounts of reducing agents and acid (concentrated phosphoric acid) were carefully balanced not only to avoid fast hydrolysis of the boranes, but also to maintain a sufficiently low pH to stabilize reduced rhenium intermediates. The preparations resulted in yields >85 % of the desired precursor complex. In addition, perrhenate (7±3 %), colloidal 188ReO2 (<5 %), and a by-product of unknown composition were also detected.

To increase the yields even further, we evaluated the recently described borohydride exchange resin (BER) as an additional reducing agent and an anion scavenger.10

EXPERIMENTAL PROCEDURES

Unless otherwise stated, all chemicals were reagent grade and used without further purification. Sodium perrhenate (Na188ReO4) was eluted from a 188W/188Re generator (Oak Ridge National Laboratory, Oak Ridge, TN, USA) using 0.9% saline. For the separation of [188Re(OH2)3(CO)3]+, HPLC analyses were performed on a WATERS system equipped with a radiometric detector using a reversed phase XTerraTM RP18 5 μm column (4.6×250 mm, Waters, Ireland). HPLC solvents consisted of methanol (solvent A) and 0.05 M TEAP (tetaethylammonium phosphate) buffer, pH 2.25, solvent B). HPLC gradient: 0-5 min: a linear gradient to 0% A/ 100% B from 100% A/ 0% B; 5-8 min: a linear gradient to 25% A/ 75% B from 0% A/ 100% B; 8-11 min: a linear gradient to 34% A/ 66% B from 25% A/ 75% B; 11-22 min: a linear gradient to 100% A/ 0% B from 34% A/ 66% B; 22-24 min: a linear gradient to 0% A/ 100% B from 100% A/ 0% B. The flow rate was 1 mL per min. For the separation of 188Re(I) tricarbonyl histidine, HPLC analyses were performed on a PERKIN ELMER system equipped with a radiometric detector (IsoScan LC gamma, Biostep, Germany) using a Hypersil ODS column (packing material 10 μm, 250× 4 mm, KNAUER, Berlin, Germany). HPLC solvents consisted of ethanol (solvent A) and 0.05 M TEAP buffer, pH 1.95 (solvent B). HPLC gradient: 0-10 min: a linear gradient to 100% A/ 0% B from 0% A/ 100% B; 10-20 min: 100% A; 20-25 min: a linear gradient to 0% A/ 100% B from 100% A/ 0% B. The flow rate was 1 mL per min. All solvents for the mobile phase were of HPLC grade and pre-filtered with 0.2 μm pore size bottle filter. The instant thin-layer chromatography (ITLC) analyses were performed on a TLC analysis system equipped with a
gamma-ray scanner (RITA TLC Analysis, Raytest, Germany). Thin layer chromatography (TLC) was performed using either silica gel plates (60F254, Merck, Darmstadt, Germany) and 99% methanol/1% conc. HCl as an eluting solvent or paper (Whatman No. 1) and 99.5% methanol/0.5% 6 M HCl as an eluting solvent.

Synthesis and Radiolabeling. \(^{188}\text{Re}(\text{I})\) tricarbonyl precursor (1) \([^{188}\text{Re(OH}_2)_3\text{(CO)}_3]^+\). A 10 mL vial containing tetrahydroborate exchange resin (BER, 3 mg), borane-ammonia \((\text{BH}_3\cdot\text{NH}_3, 3 \text{ mg})\) and potassium boranocarbonate \((\text{K}_2[\text{H}_3\text{BCO}_2], 3 \text{ mg})\) was capped with a rubber stopper. A solution of 1 ml of sodium perrhenate \((\text{Na}^{188}\text{ReO}_4)\) with up to 50 MBq and 7 \(\mu\text{L}\) of concentrated phosphoric acid (85%) was added to the vial by a 20 mL syringe and the was then heated to 60 oC in a boiling water bath for 15 min. Pressure from the evolving gas (about 10 mL) was balanced with the syringe. After cooling to room temperature, the neutral \(^{188}\text{Re}(\text{I})\) tricarbonyl precursor was obtained (Scheme 1). Yield: >97% determined by means of HPLC and TLC.

\[
\text{[}^{188}\text{ReO}_4^- \text{]} \xrightarrow{i) \text{Conc. H}_3\text{PO}_4} \text{ii) BER, BH}_3\cdot\text{NH}_3, \text{K}_2[\text{H}_3\text{BCO}_2], \text{60 oC, 15 min} \text{]} \rightarrow \begin{array}{c}
\text{OH}_2 \\
\text{OC} \\
\text{OH}_2 \\
\text{CO} \\
\end{array} + \begin{array}{c}
\text{HN} \\
\text{OC} \\
\text{HN} \\
\text{O} \\
\end{array}
\]

Scheme 1. Synthesis of \(^{188}\text{Re}(\text{I})\) tricarbonyl precursor (1) and \(^{188}\text{Re}(\text{I})\) tricarbonyl histidine (2)

\(^{188}\text{Re}(\text{I})\) tricarbonyl histidine (2). A 10 mL vial containing histidine (500 \(\mu\text{L}\)) was capped with a rubber stopper. A solution of 800 \(\mu\text{L}\) of \([^{188}\text{Re(OH}_2)_3\text{(CO)}_3]^+\) in saline with up to 50 MBq was added to the vial using a 1 mL syringe. The reaction vial was sealed and heated to 75 oC for 30 min. After 30 min, the reaction mixture was cooled to room temperature (Scheme 1). Yield: >97% determined by means of HPLC and TLC.

Tetrahydroborate exchange resin (BER). The tetrahydroborate exchange resin (BER) as a reducing agent was prepared by the reported method. Chloride-form resin (Amberlite\(^{8}\) ion exchange resin, 12.5 g) was slurry-packed with water into a 30-mL fritted glass funnel mounted on a filter flask. An aqueous sodium tetrahydroborate solution (200 mL, 0.25 M) was slowly passed through the resin over a period of 30 minutes. The resulting resins were washed thoroughly with distilled water until free of excessive NaBH\(_4\), and finally with ethanol. The tetrahydroborate-form anion exchange resin was then partially air-dried by removing ethanol on the surface of the BER. This resin was analyzed for its tetrahydroborate content by hydrogen evolution upon an acidification with 0.08 M HCl, and the average capacity of BER was found to be 2.5 meq of tetrahydroborate ion per gram of resin.

RESULTS

The HPLC chromatogram of \(^{188}\text{Re}(\text{I})\) tricarbonyl precursor 1 and \(^{188}\text{ReO}_4^-\) showed that retention times of those species are 4.7 and 9.8 min, respectively. The retention time of the \(^{188}\text{Re}(\text{I})\) tricarbonyl precursor was compared to that of \(^{99m}\text{Tc}(\text{I})\) tricarbonyl precursor and were found to be identical. The radiolabeling yield of \(^{188}\text{Re}(\text{I})\) tricarbonyl precursor in the reaction mixture was determined by the HPLC analysis and found to be > 99%. The HPLC
chromatogram of a typical $^{188}$Re(I) tricarbonyl precursor in the reaction mixture is shown in Figure 1. The complex is stable (>95%) for approximately 3 h. After this time, decomposition of the complex was observed. Paper electrophoresis investigations in aqueous solution confirmed the cationic charge of $^{188}$Re(I) tricarbonyl precursor in a neutral solution. The assay for the formation of the $^{188}$Re(I) tricarbonyl precursor, reduced hydrolyzed $^{188}$Re, and $[^{188}\text{Re}]$perhenate ion was achieved by investigating their positions using an instant thin-layer chromatography (ITLC).

Fig. 1. HPLC Chromatograms of $^{188}$Re(I) tricarbonyl precursor and Na$^{188}$ReO$_4$

$^{188}$Re(I) tricarbonyl precursor: >95% (Rf = 0.4); reduced hydrolyzed $^{188}$Re: less than 3% (origin); $[^{188}\text{Re}]$perhenate ion: 0% (Rf = 0.8). The HPLC chromatogram of $^{188}$Re (I) tricarbonyl histidine showed that the retention time of the complex is 11.4 min. The radiolabeling yield of $^{188}$Re(I) tricarbonyl histidine in the reaction mixture was determined by the HPLC analysis and found to be >97%. The HPLC chromatogram of $^{188}$Re(I) tricarbonyl histidine (2) in the reaction mixture is shown in Figure 2. The retention time of the $^{188}$Re(I) tricarbonyl histidine was compared to that of $^{99m}$Tc(I) tricarbonyl histidine and they were found to be identical.
DISCUSSION

The tetrahydroborate exchange resin (BER) contains tetrahydroborate ion (BH$_4^-$) bound to the cation which is supported on the polystyrene matrix, and the cation has a quaternary alkyl ammonium functionality used for adhering the tetrahydroborate ion or negatively charged species. A schematic illustration of the use of BER is shown in Figure 3.

![Fig. 3. BER as a solid-phase reducing agent and an anion scavenger](image)

The results of the present study demonstrate that employing the borohydride exchange resin (BER) as a novel reducing agent and an anion scavenger. The $^{188}$Re(I) tricarbonyl precursor having high radiochemical purity and labeling efficiency can be prepared without nitrogen gas flushing and ice bath cooling compared to the conventional method. The BER is advantageous in terms of being stable over a wide range of pH values (2-11) and applicable to biologically active molecules, as well as being easily removable through filtration when being administrated. This approach thus provides the potential for economic and effective production of $^{188}$Re(I) tricarbonyl precursor without the formation of unreacted $^{188}$ReO$_4^-$, colloidal $^{188}$ReO$_2$, and negatively charged impurities by reinforcing the conventional reducing agent requiring very stringent condition for preparation.
ACKNOWLEDGMENTS

This work was supported by the Nuclear R & D Program of the Korean Ministry of Science and Technology and the Technical Cooperation Program of the International Atomic Energy Agency.

REFERENCES


Country report: Pakistan

Second Research Co-ordination Meeting on Development of Radiopharmaceuticals Based on $^{188}\text{Re}$ and $^{90}\text{Y}$ for Radionuclide Therapy, 22-26 March 2010, Vienna

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Experimental

Materials and methods

All the chemicals used in the experiments were of analytical reagent grade, and were purchased from E. Merck (Germany). Yttrium oxide powder and Strontium carbonate were product of Johnson and Mathey. Commercially available EDTMP was obtained from Dojin Laboratories (Kumamoto, Japan). Whatman 3 MM chromatography paper was used for ascending paper chromatography and paper electrophoresis studies. Membrane filters were obtained from Millipore USA. Strontium-90 was purchased from Amersham UK.

Production of, Strontium-85 and yttrium-90

Known Quantities of yttrium oxide/strontium carbonate targets were sealed in quartz ampoules and cold welded into aluminum cans. The irradiations were carried out inside the core of the 10 MW swimming pool type Pakistan Research reactor-I (PARR-I) for up to 120 hours at a neutron flux of $\sim 1.5 \times 10^{14} \text{ cm}^{-2} \text{ s}^{-1}$. The irradiated material was dissolved in concentrated hydrochloric acid, evaporated and taken in distilled water.

Activity measurement

Yttrium-90 samples were measured in an ionization chamber (Capintec CRC 15R, USA) when used at the megabecquerel (MBq) level and with a beta counter, when used at lower levels. The $^{90}\text{Sr}$ was also measured by beta counter. The $^{85}\text{Sr}$ was measured by gamma spectrometry on an HPGe solid detector. The HPGe spectrometric system was calibrated using standard calibration radioactive sealed sources.

Filtration of $^{90}\text{Y}$ colloid by glass wool

Approximately 10 mCi of the radioactive solution was taken in 1 M HCl. The pH of solution was adjusted to 12 with concentrated NaOH and filtered slowly through a small glass wool column. The glass wool then washed with 1 N NaOH and washed again with more dilute NaOH of pH 9.00. one N HCL was then slowly passed through glass frit. In order to eliminate a possibility of the presence of any trace quantity of $^{90}\text{Sr}$ in the filtrate, the procedure mentioned above was repeated. The resulting filtrate was shown to contain only $^{90}\text{Y}$ as evidenced by a half-life.

Filtration of $^{90}\text{Y}$ colloid by membrane filter

The present investigation was planned to study the state of carrier free $^{90}\text{Y}$ in filtration process with membrane filters. This type of filters is effectively impermeable to particles of colloid
size, and by the constant area and uniform pore size of the filters, it would be expected to obtain more quantitative results.

The chloride solution of $^{90}$Sr in equilibrium with $^{90}$Y was neutralized with NH$_4$OH and passed through Millipore bacteria filter papers. Washing was performed by 1 M NH$_4$OH. Removal of $^{90}$Y was achieved by 1 M HCl.

**Preparation of $^{90}$Y-EDTMP**

EDTMP was dissolved in distilled water or dilute NaOH. $^{90}$Y chloride solution was added to the EDTMP solution. The pH was adjusted to 8.

**Paper chromatography**

Five microlitre (µl) of the test solution was spotted at 2 cm from one end (bottom end) of Whatman 3 MM chromatography paper strips (14 x 2 cm). The strips were developed in pyridine/ethanol/water (1:2:4), dried, cut into 1 cm segments and the activity was measured. Sometimes strips were scanned by 2π Scanner (Berthold, Germany).

**Results and Discussion**

Various experiments were carried out for the separation of $^{90}$Y from $^{90}$Sr using colloid formation behavior of $^{90}$Y in basic pH range. When the acidic solution of $^{90}$Sr/$^{90}$Y was treated with NaOH and passed through a small glass wool column, more than 95% $^{90}$Y was retained on glass wool, which was further washed with 0.1 M NaOH. The $^{90}$Y was extracted in 1 M HCl. Radionuclidic purity was determined by half life measurement. The contamination of parent was less than 0.0001 %. Similarly when the colloid was passed through membrane filter more than 95% $^{90}$Y was retained which was recovered by 1 M HCl. The contamination of $^{90}$Sr was less than 0.0001 %.

The stock solution of $^{90}$Sr/$^{90}$Y in NH$_4$OH/NaOH was again passed through Millipore filter paper or glass wool after one week. The retention of $^{90}$Y was insignificant. The equilibrium mixture of $^{90}$Sr/$^{90}$Y was acidified with HCl and again precipitated with addition of NH$_4$OH or NaOH. The basic solution was passed through the Millipore filter or glass wool. More than 90% activity of $^{90}$Y was retained, which was finally extracted in 1 M HCl. It was concluded that for recovery of $^{90}$Y from $^{90}$Sr by colloid formation, the acidic solution of $^{90}$Sr/$^{90}$Y shall be freshly precipitated by NH$_4$OH or NaOH.

It was also observed that the adsorption of $^{90}$Y was quite high when the $^{90}$Sr solution was left in a glass vial for the growth of $^{90}$Y. However it can be easily extracted after keeping it in boiling water bath for few minutes.

The separated $^{90}$Y via colloid formation was used for the labeling of EDTMP. Labeling efficiency of $^{90}$Y-EDTMP was more than 98%.
Country report: Poland

The program of the CRP in the first 18 months was focusing on 4 subprojects, which are reported below individually.

1. DETERMINATION OF $^{90}$Sr IN $^{90}$YCl$_3$ SOLUTION USING DGA AND SR-SPEC RESINS (EXTRACTION CHROMATOGRAPHY) AND BY TLC ACCORDING TO USP.

D. Pawlak, R. Mikolajczak, A. Korsak, T. Dziel, J.L. Parus

Extraction chromatography:

**DGA column.**
$^{85}$SrCl$_2$ (1740 Bq) in 1 ml 3M HNO$_3$ with carrier YCl$_2$ (0,005 μg)
Two 1 ml fractions eluted with 3M HNO$_3$, 5 fractions eluted with 0,1M HNO$_3$.

**Sr-spec column.**
$^{85}$SrCl$_2$ (1740 Bq) in 1 ml 3M HNO$_3$ with carrier YCl$_2$ (0,005 μg)
Four 1 ml fractions eluted with 0,1 M HNO$_3$, 7 fractions eluted with 0,05 M HNO$_3$.

Paper chromatography:

On the 20cm x 2cm strip of Wh P81 (cation-exchange paper) 50 μl of carrier solution was placed (0,34 mg/ml YCl$_3$ . 6H$_2$O and 0,30 mg/ml SrCl$_3$ . 6H$_2$O) and dried. At the same spot 5 μl of tested solution was placed, chromatography developed in 3M HCl.

Time of development was about 1,25 h (front of developing solution moves about 13.5 cm from origin. Strips were dried, 7 cm upper part was cut and measured in Liquid Scintillation Counter.

- Calibrated solutions of $^{85}$Sr and $^{90}$Sr+$^{90}$Y
- LSC spectrometer WALLAC 1411
- Ultima Gold liquid scintillator

Results

 Extraction chromatography

Fig. 1. Elution profiles of $^{85}$SrCl$_2$ from DGA and Sr-spec columns
Elution field of $^{85}\text{Sr}$ from DGA column was 100.7% and elution field of $^{85}\text{Sr}$ from Sr-Spec column was 96.2%.

**Paper chromatography**

Fig. 2. Radioactivity distribution of $^{85}\text{Sr}$ on the strip

$^{90}\text{Sr} + ^{90}\text{Y}$ (solution in equilibrium), radioactivity applied on the strip around 58 kBq $^{90}\text{Sr}$, experiment performed in triplicate. Radioactivity measured after chromatography: 65.7 kBq, 66.9 kBq, 65.5 kBq.

Fig. 3. Radioactivity distribution of $^{90}\text{Sr} + ^{90}\text{Y}$ (solution in equilibrium)

$^{90}\text{Y}$ solution (production day), radioactivity applied on the strip around 470 MBq.
The same strips were stored dry for 3 weeks to permit decay of $^{90}\text{Y}$ and again measured.

Fig. 4. Radioactivity distribution of the $^{90}\text{Y}$ solution (production day) Below – respective beta spectrum

Fig. 5. Radioactivity distribution of the $^{90}\text{Y}$ solution measured after 3 weeks of cooling
Conclusions

1. It seems that strontium can be more efficiently eluted for the DGA resin than form the Sr-spec resin.

2. Paper chromatography (USP) method provides good resolution of Y and Sr, more results are needed to evaluate results statistically.

3. Comparison of paper chromatography (USP) with paper extraction chromatography was not possible due to the problems in purchasing of the KSM-17.

4. Further works in order to determine detection and determination limits of each of proposed methods are planned.


Monograph “Yttrium Y\textsuperscript{90} Ibritumomab Tiuxetan Injection” p. 3487.

**Radionuclidic purity** (Content of \textsuperscript{90}Sr in an yttrium Y 90 chloride solution) – Prepare a strontium/yttrium carrier solution containing 0.34 mg of yttrium chloride (YCl\textsubscript{3}x6H\textsubscript{2}O) and 0.30 mg of strontium chloride (SrCl\textsubscript{2}x6H\textsubscript{2}O) per mL of 0.1 N hydrochloric acid. Apply about 50 µL of this solution at the origin of a 2 x 19-cm cellulose phosphate chromatographic strip (see Chromatography 〈621〉), and allow to dry. Apply about 5 ml of the yttrium Y 90 chloride radiolabelling solution at the origin, and develop the chromatogram by ascending chromatography over a period of about 1.25 hours, using 3N hydrochloric acid as the developing solvent, until the solvent migrates to the 15-cm mark. Allow to dry. Cut the strip at the 8-cm mark, and place the upper section (solvent front) in a suitable liquid scintillation solvent. Using a suitable counting assembly (see Beta-Emitting Radionuclides in the Assay section of Identification and Assay of Radionuclides under Radioactivity 〈821〉), determine the radioactivity, in kBq (or µCi) per mL of yttrium Y 90 chloride solution.

The total radioactivity of \textsuperscript{90}Sr is not greater than 740 kBq per 37 GBq (or 20 µCi per Ci) of \textsuperscript{90}Y at the expiration date as stated on the labeling.

**Ascending chromatography using the cation-exchange paper Wh P81**

The procedure recommended by USP for the determination of \textsuperscript{90}Sr in \textsuperscript{90}Y at max. radioactivity ratio of 1 to 50 000 (740 kBq \textsuperscript{90}Sr per 1 Ci \textsuperscript{90}Y) has been used.

In this procedure the carrier solutions are used containing 0.34 mg yttrium chloride (YCl\textsubscript{3}x6H\textsubscript{2}O) and 0.30 mg strontium chloride (SrCl\textsubscript{2}x6H\textsubscript{2}O) in 1 ml 0.1 N HCl.

When using \textsuperscript{85}Sr as a tracer, the measurements showed recovery of Sr at about 60%, while the use of \textsuperscript{90}Sr + \textsuperscript{90}Y resulted in \textsuperscript{90}Sr and \textsuperscript{90}Y recovery close to 100% for 2 strips and significantly lower for \textsuperscript{90}Y and close to 0 for \textsuperscript{90}Sr for another 2 strips, indicating some mistakes in performing the experiment.

The series of 5 experiments were performed using 222.8 kBq \textsuperscript{90}YCl\textsubscript{3} – with addition of varying \textsuperscript{90}Sr radioactivity – in the following quantities 0, 8.9, 17.8, 35.6 and 44.5 Bq; recovery was 0, 2.4, 13.4, 15.0 and 24.1 Bq respectively, the recovery was not reproducible and generally low. The radioactivity of 8.9 Bq corresponded to the radioactivity ratio of 1 to
25 000. Another series of experiments with the $^{90}$Y radioactivity of 159.1 kBq also resulted in low recovery and non reproducible results.

On the other hand, when the samples of $^{90}$Sr + $^{90}$Y standard solutions were prepared by volume (0.85, 1.27, 2.29, 5.08 and 6.78 μl), the reproducibility of measurements was good but the recovery was much over 100% - at the level of 137% for the maximal content of $^{90}$Sr + $^{90}$Y.

**Conclusion:** the procedure still needs improvements and will be developed in the next months.

**Column chromatography with DGA resin**

Glass or plastic column with the resin bed of 2 cm high and 0.5 cm diameter, size of grains from 50 to 100 μm. $^{85}$SrCl₂ solution in 3 M HNO₃, elution with 0.1 M HNO₃. Recovery between 98 and 108%. The Y eluted from this column with 0.1 M HCl. The series of experiments was performed with varying radioactivity ratio of $^{90}$Sr : $^{90}$Y of 1:1, 1:10, 1:100, 1:1000, 1:10 000, 1:100 000, 1:500 000 and 1:1 000 000.

Samples were prepared in 3 M HNO₃ with YCl₃ carrier of 0.025 mg in 1 ml volume. Columns were eluted using 1 ml fractions of 3 M HNO₃. $^{90}$Sr was eluted with 6 ml 0.1 M HNO₃, $^{90}$Y -8 ml 0.1 M HCl.

Radioactivity of $^{90}$Sr in equilibrium with $^{90}$Y was 200 Bq.

<table>
<thead>
<tr>
<th>Radioactivity ratio $^{90}$Sr:$^{90}$Y</th>
<th>$^{90}$Sr recovery [Bq]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>205</td>
</tr>
<tr>
<td>1:10</td>
<td>223</td>
</tr>
<tr>
<td>1:100</td>
<td>213</td>
</tr>
<tr>
<td>1:1 000</td>
<td>209</td>
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<td>1:10 000</td>
<td>223</td>
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<tr>
<td>1:100 000</td>
<td>199</td>
</tr>
<tr>
<td>1:500 000</td>
<td>185</td>
</tr>
<tr>
<td>1:1 000 000</td>
<td>215</td>
</tr>
</tbody>
</table>

Chromatography with tandem of columns – with Sr-Spec and DGA resin

$^{90}$Sr was washed from the Sr-Spec column with 0.05 m HNO₃ in 6 ml volume, then acidified with concentrated HNO₃ to around 3 M and loaded on DGA column, afterwards the $^{90}$Sr was eluted from the column with 0.1 M HNO₃.

<table>
<thead>
<tr>
<th>Radioactivity ratio $^{90}$Sr:$^{90}$Y</th>
<th>Recovery $^{90}$Sr [Bq]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10 000</td>
<td>64 (32%)</td>
</tr>
<tr>
<td>1:10 000 000</td>
<td>89 (44.5%)</td>
</tr>
</tbody>
</table>

The measurement of yttrium solution from the production batch: 230, 248 i 307 Bq – taking into account the average yield of 72%, the content of $^{90}$Sr was 1.82×10⁻⁴%. For the same production batch tested at the QC group using the Sr-Spec column the determined content of $^{90}$Sr was 2.4×10⁻⁴ %.

2. PRELIMINARY INVESTIGATION ON ANTIBODY BIOTINYULATION

E. Byszewska-Szpocinska, E. Jakubowska, R. Mikolajczak
The preliminary investigation on antibody biotinylation has been performed using commercially available polyclonal human immunoglobulin IgG, mw 150 kDa (Biomed Sp.z o.o, Lublin). Sulfo-NHS–Biotin (Pierce Biotechnology) (Fig.1) was used as biotinylating agent. Activated biotin as sulfosuccinimidyl-6-[biotin-amido]hexanoate. is one of the commonly used reagent for proteins biotinylation. Sulfo-NHS-biotin effectively reacts at pH 7-9 with primary amino groups (- NH$_2$) of proteins provided by lysine residues and N-terminus of polypeptide, forming stable amide bond (Fig. 2). This reagent dissolves well in water.

![Fig. 1. Sulfo-NHS–Biotin (sulfosuccinimidyl-6-[biotin-amido]hexanoate )](image)

Sulfo-NHS-Biotin + primary amino group of protein $\rightarrow$ biotinylated protein + Sulfo-NHS group (removed by dialysis)

![Fig. 2. Biotinylation of proteins using Sulfo-NHS-Biotin](image)

Biotinylation depends on the number of free amine residues in the protein molecule. In diluted solutions biotinylation requires high molar excess of reagent. In standard conditions, for biotinylation of antibody at concentration of 1-10 mg/ml 20-fold molar excess of biotinylated reagent is used, which is expected to result in 4 -6 biotin groups substitution per antibody molecule (mw 150 kDa ).

**Biotinylation of human polyclonal IgG**

Fresh solution of Sulfo-NHS-Biotin (2,2mg in 500μl) was prepared directly prior to its use in de-ionized water. 1 ml of IgG (2mg/ml in PBS(0,1M phosphate buffer + 0,15M sodium chloride, pH 7,2) was reacted with 26,8 μl of Sulfo-NHS-Biotin solution (20-fold molar excess). Reaction mixture was incubated for 1 h at RT. Non-reacted and hydrolyzed biotinylated reagent was removed by dialysis to 200-fold larger volume of PBS, carried out at 2-8°C and changing the buffer 4 times. After dialysis the product solution was filtered using 0,22μm (Millipore). The effect of antibody biotinylation was evaluated by adding an aliquot of biotinylated antibody to the mixture of HABA (hydroxyazobenzene-2 carboxylic acid)with avidin. HABA binds to avidin forming the complex which absorbs light at 500nm, the coefficient of extinction $\epsilon = 35 500$ M$^{-1}$cm$^{-1}$. The method of determination is based on the
decrease of the [(HABA)₄: Avidin complex absorbance, when HABA is replaced by biotin. (Fig. 3.)

![Diagram](image)

Fig. 3. Schematic presentation of method for determination of biotin bound to protein.

The reagent containing 1 mg of avidin and 60 μl 10mM HABA (Pierce) in 1,94 ml PBS was prepared. The microplate reader was used and the 96-wells microplates. The aliquots of 180 μl of this reagent were pipeted to the wells, the absorbance measured at 500nm (average A₅₀₀ H/A = 0.691), then to the same wells 20 μl samples of biotinylated antibody solution were added, followed by measurement of absorbance at 500nm (average A₅₀₀ H/A/B = 0.613).

Taking into account the above measured absorbance values, extinction coefficient ε, light pathway in microplates (0.5 cm), concentration of antibody expressed in mols, dilution factor (10x) the number of biotin mmols per mmol of IgG antibody taken for reaction was calculated to be 3.5 (3.5 molecules of biotin per IgG molecule).

**Biotinylation of monoclonal antibody fragment p 804 (mw 30 kDa)**

The method of bitinylation was tested using antitubulin monoclonal antibody fragment (scFvTU-20, mw 30 kDa), obtained from Institute of Molecular Genetics, Prague, Czech Republic. Solution of this antibody fragment was dialysed to PBS (as described for IgG) and then concentrated using Microcon filters (Millipore) to obtain 1.2 mg/ml concentration. The biotinylation procedure was performed for 1 mg of this protein in the same way as described for IgG and using 20-fold molar excess of Sulfo-NHS-biotin (72.5 μl). After 1 h incubation at RT the PBS dialysis was performed. The determination of the number of biotins conjugated to the protein was performed using HABA/avidin method. The calculated result was 1.55 biotin molecules per molecule of p 804 fragment.

The above described method of biotinylation will be further used for other monoclonal antibodies and their fragments.

**3. THE DEVELOPMENT OF METHOD FOR PREPARATION OF HUMAN ALBUMIN MICROSPHERES AS POTENTIAL RADIONUCLIDE CARRIERS FOR DIAGNOSTIC AND THERAPEUTIC USE**

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**Introduction**

Human serum albumin microspheres (HAM) of various size have been widely used for clinical nuclear medicine as carriers for radioactive diagnostic and therapeutic molecules since 1969 [1,2]. Their widest application has been found for scanning of lungs in the diagnosis of pulmonary embolism, pulmonary infraction and other certain lung disorders. The
The purpose of this study was to develop and establish a methodology for production of albumin microspheres batches of 10-32 μm size range with reproducible physical and biological properties suitable for labelling with a variety of radionuclides in accordance with the requirements for Active Pharmaceuticals Ingredient (API).

**Methods**

**Preparation of HAM**

In the present study we investigated a novel method of HAM production using emulsification and heat stabilization technique described previously [3,4]. In brief, HSA solution with SDS was added to liquid paraffin containing SDS and Tween 80. The mixture was stirred in order to obtain water/oil emulsion. The emulsion was then heated to allow the formation and solidification of microspheres. The oil was removed by decantation and microspheres were washed with diethyl ether. The particles were then dried in vacuum and later sieved. Ten batches of microspheres ranging in diameter from 10 to 32 μm were prepared by the above described method. The particle size analysis was performed by optical microscopy using a light microscope equipped with an ocular micrometer and a light camera (Fig.1). The microspheres were sized and photographed in normal saline containing Tween 80 to prevent aggregation. The particles in each prepared batch were measured using a calibrated ocular micrometer and a specific computer program. The contribution of microspheres particles in selected size range (in percent) was determined by weight analysis (Table 1).

<table>
<thead>
<tr>
<th>Nr of batch</th>
<th>Contribution of HAM fractions [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size range [μm]</td>
<td>&lt;10</td>
</tr>
<tr>
<td>1</td>
<td>0.9</td>
</tr>
<tr>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>3</td>
<td>0.3</td>
</tr>
<tr>
<td>Average</td>
<td>0.5</td>
</tr>
</tbody>
</table>

**Labelling of the Microspheres**

Preliminary study of HAM labelling with $^{99m}$Tc, $^{90}$Y and $^{177}$Lu was performed for confirmation of particles usefulness as radionuclides carriers. The labelling process with $^{99m}$Tc was carried out using the direct method. HAM were coated with SnCl$_2 \times 2$H$_2$O and further labelled with $^{99m}$Tc (150 MBq) in 0.9 % saline solution. The suspension was incubated at room temperature for 30 min with shaking. $^{99m}$Tc-HAM were separated from non-bound pertechnetate by filtration.
For HAM labelling with $^{90}$Y and $^{177}$Lu the particles were modified by covalent attachment of suitable chelators onto the surface of the spheres. Modified microspheres (X-HAM) were labelled in conventional conditions. X-HAM were suspended in ascorbic buffer (pH 4.5) and approx. 50 MBq $^{90}$Y (70 MBq $^{177}$Lu) was added. Then the vial was heated to 90°C and incubated for 30 min with shaking. $^{90}$Y ($^{177}$Lu)-X-HAM were separated by filtration.

**In Vitro Stability of $^{90}$Y ($^{177}$Lu)-X-HAM**

To estimate the in vitro stability of the radiolabelled complexes, samples of $^{90}$Y–X-HAM and $^{177}$Lu-X-HAM were incubated with human plasma for 48 h in 37 °C. Aliquots of the suspension after centrifugation were removed after 1, 24, 48 h and particle-associated radioactivity was calculated (Fig. 2).

Fig. 2. In vitro stability of $^{90}$Y–X-HAM and $^{177}$Lu-X-HAM

**Results and Conclusion**

The average production yield of HAM for receiving the desired size range between 10-32 μm amounts to 84 % and the mean size of particles was estimated to about 15 μm. Optical micrographs show microspheres as very regular spherical forms with quite smooth surfaces and rather narrow spread of size in the selected range.

The labelling yields determined as relation of measured radioactivity remaining in the membrane filter to the total radioactivity were > 95% for $^{99}$mTc-HAM complex and > 98% for $^{90}$Y–X-HAM and $^{177}$Lu-X-HAM complexes. Labelled modified microspheres showed high in vitro stability in human plasma with only 4-5% loss of radioactivity from the surface after 48 h of incubation.
Biodistribution of $^{177}$Lu-HMA

15 min p.i. over 90% of radioactivity accumulates in lungs (>80%) required by Pharmacopoeia). The uptake in liver and bones increases after 4 and 24 h p.i. which might indicate $^{177}$Lu dissociation. Further investigations of $^{177}$Lu and $^{90}$Y in vivo stability are in progress.

$^{188}$Re-HMA for hepatic cancer treatment

Clinical trial has been initiated at the Clinic in Warsaw using commercially available HMA/SnCl2 (from ROTOP, Germany). Polatom assisted the clinic in labelling technique and preparation of intermediate solutions. The clinic is interested to use $^{188}$Re-HMA or $^{90}$Y/$^{177}$Lu HMA developed within the CRP.

References


4. PREPARATION OF RADIOCOLLOIDS FOR RADIOSYNOVECTOMY USING RADIONUCLIDES OF VARIOUS BETA ENERGIES

J.L.Parus, W. Mikolajczak, R.Mikolajczak

The aim of the project is synthesis of monodispersive particles of yttrium citrate and characterization of its surface with instrumental techniques (collaboration with Maria Curie-Sklodowska University in Lublin, prof. W. Janusz). In this report short information on methods involved is provided. The same approach was initiated to obtain and characterize $^{188}$Re sulphide colloid.
Preparation of $^{90}$Y citrate colloid

Particle size distribution was investigated as a function of chemical composition in wide range of concentrations and depending on pH (5.0, 5.5, 6.0, 6.5, 7.0). Stable size distribution was obtained for pH 6.5 - 7.0.

Composition of the preparation: 2mM yttrium nitrate (150ml), 10mM sodium citrate (10ml). Water for injection (240ml), adjusted to pH 6 using 0.1 M NaOH, incubation in water bath for 1h. Yield 0.5 to 1.0 g.

Fig. 1. Surface of yttrium citrate made using AFM microscope (Atomic Forces Microscope)

Crystals of yttrium citrate were also observed under the scanning electron microscope (SEM).

Fig. 2. Size distribution of yttrium colloid obtained with ZetaSizer Nano ZS (Malvern Inst)
Development, Preparing and Quality Assurance of Radiopharmaceuticals Based on $^{188}$Re and $^{90}$Y for Radionuclide Therapy: The Possibilities for their Production in Laboratory for Radioisotopes, Ins «Vinča»

Divna Djokić

Vinča Institute of Nuclear Sciences, Laboratory for Radioisotopes, Belgrade, Serbia

Time period covered: 2008-07-1 to 2009-12-31

The main object of the research planned for this project was to optimize the procedures for the $^{90}$Y and $^{188}$Re labelling of different compounds as well as their in vitro and in vivo evaluation. The work has been involved setting up the facilities, standardization of preparing protocols, and improving existing quality assurance/quality control (QA/QC) procedures in order to supply reliable products to the national nuclear medicine community.

The planed program of work (Research Contract No. 14827/R0, for the period 2008-04-01 to 2009-03-31) was:

1. Radiolabelling of carrier molecules such as amino acids and peptides with Y-90 and Re-188;
2. Development of Y-90 particulates;

The IAEA renewed the Contract (Contract No. 14827/R0 for the period 2009-03-17 to 2010-03-16) with the following modifications of the Program of work:

1. Development of Sr-90/Y-90 electrochemical generator and QC of Y-90;

This presentation highlighting the work done through the Research contract No: 14827 (14827/R0, 14827/R1) during the first 18 months.

This work could be presented in three separated parts:

1. $^{90}$Y and $^{186/188}$Re complexes for tumor therapy and bone palliation:
   1) $^{90}$Y complexes of phosphonate ligands of HEDP, MDP and DPD;
   2) $^{90}$Y complexes of DMSA;
2. $^{90}$Y-particulates
   1) $^{90}$Y-colloids for hepatocellular carcinoma: $^{90}$Y-Sb$_2$S$_3$ and $^{90}$Y-Sn colloids;
   2) $^{90}$Y-colloids colloids for radiosynovectomy: $^{90}$Y-HA without or with presence of stabilizers like phosphonates HEDP, MDP, DPD;
3. Development of Sr-90/Y-90 generator: electrochemical \(^{90}\)Sr/\(^{90}\)Y - generator and QC of \(^{90}\)Y.

1. \(^{90}\)Y complexes for tumor therapy and bone palliation

The use of the therapeutic radiopharmaceuticals that localize selectively at the metastasis sites is found to be an effective treatment for the palliation of pain. Radionuclides decaying by the emission of \(\beta\)-particles like yttrium-90 are preferred in most of this applications. \(^{90}\)Y have favorable properties for therapy, Table 1:

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Half life (days)</th>
<th>Emission</th>
<th>Max. range (mm) (air/tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y-90</td>
<td>2.67</td>
<td>(\beta)-</td>
<td>12/3.9</td>
</tr>
</tbody>
</table>

1) \(^{90}\)Y complexes of HEDP, MDP and DPD

The object of these studies was to research the possibility for \(^{90}\)Y-complexion of polyphosphonate ligands trihydroxy (phosphonomethyl) phosphanium (MDP), (1-hydroxy-1-phosphonoethyl)phosphonic acid (HEDP) and (2 hydroxy-3,4-dioxopentyl) phosphate (DPD). Molecular structures of polyphosphonate ligands MDP, HEDP and DPD are presented in Fig.1.

![Molecular structures of polyphosphonate ligands HEDP, MDP and DPD](image)

The labelling of polyphosphonate ligands was carried out by varying experimental parameters such as: ligand concentration, pH values, time and temperature of the reaction. Analysis of the complexes enclosed the radiochemical quality control methods (ITLC, paper chromatography and HPLC), in vitro stability studies of the \(^{90}\)Y-complexes, as well as physiological distribution studies per organ of experimental animals (health male Wistar rats).

The preparation of \(^{90}\)Y-HEDP, \(^{90}\)Y-MDP and \(^{90}\)Y-DPD

\(^{90}\)Y-labelling: A direct labeling method was optimized by varying HEDP, MDP and DPD concentration and pH in the labeling mixture, as well as the reaction temperature and the reaction time. The stock solution of polyphosphonate ligands was prepared by dissolution the desired ligand concentration (0.01–10 mg/ml) in double distilled water. An appropriate amount of the \(^{90}\)Y-chloride solution (~370 MBq per vial) was edded. The pH values of the resulting reaction mixtures were adjusted to 4.0 and/or 6.5. The total reaction volume in each vial was maintained at 3 ml. Ascorbic acid (10 mg) was used as radiolytic stabilizer in all samples;

Radiochemical purity: ITLC-SG – 0.9 % NaCl or CH\(_3\)OH (Rf=0.9-1.0 for free \(^{90}\)Y\(^{3+}\) and Rf=0.0-0.2 for \(^{90}\)Y-complex);
Serum stability studies: The stability of $^{90}$Y–HEDP, $^{90}$Y–MDP and $^{90}$Y–DPD in human serum was assessed by measuring the release of $^{90}$Y from the complex at 37 °C over a 10-day period.

Organ distribution studies: the experiments were done as distribution per organ of animals (health male Wistar rats).

The results: obtained results have shown that radiochemical purity was > 95 % for all $^{90}$Y-complexes. The serum stability result have shown that the complex was quite stable at the studied conditions up to 10 days. No significant dissociation of activity from the complex was observed, e.g. the percentage of $^{90}$Y released from this complex at 10 days was <2.0%.

The results for organ distribution studies of $^{90}$Y-complexes were presented in Table 2.

Table 2. Organ distribution studies of $^{90}$Y–MDP, $^{90}$Y–HEDP and $^{90}$Y–DPD% (id/g ± SD)

<table>
<thead>
<tr>
<th>$^{90}$Y– complexes</th>
<th>$^{90}$Y–MDP (pH=6.5±7.5)</th>
<th>$^{90}$Y–HEDP (pH=6.5±7.5)</th>
<th>$^{90}$Y–DPD (pH=6.5±7.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organ</td>
<td>1 h</td>
<td>24 h</td>
<td>1 h</td>
</tr>
<tr>
<td>Blood</td>
<td>0.206 ± 0.065</td>
<td>0.057 ± 0.031</td>
<td>0.469 ± 0.039</td>
</tr>
<tr>
<td>Heart</td>
<td>0.172 ± 0.065</td>
<td>0.132 ± 0.044</td>
<td>0.251 ± 0.033</td>
</tr>
<tr>
<td>Lung</td>
<td>0.242 ± 0.062</td>
<td>0.124 ± 0.054</td>
<td>0.242 ± 0.077</td>
</tr>
<tr>
<td>Liver</td>
<td>5.424 ± 0.544</td>
<td>3.225 ± 0.517</td>
<td>0.253 ± 0.036</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.072 ± 0.360</td>
<td>1.424 ± 0.765</td>
<td>0.139 ± 0.049</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.317 ± 0.065</td>
<td>0.500 ± 0.191</td>
<td>0.825 ± 0.151</td>
</tr>
<tr>
<td>Intestines</td>
<td>0.054 ± 0.011</td>
<td>0.029 ± 0.011</td>
<td>0.060 ± 0.006</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.417 ± 0.205</td>
<td>0.199 ± 0.071</td>
<td>0.204 ± 0.007</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.170 ± 0.011</td>
<td>0.144 ± 0.014</td>
<td>0.227 ± 0.060</td>
</tr>
<tr>
<td>Bone</td>
<td>2.589 ± 0.334</td>
<td>6.972 ± 1.438</td>
<td>4.840 ± 0.805</td>
</tr>
</tbody>
</table>

The organ distribution study of $^{90}$Y-HEDP (Table 2) has shown that complexes were localized in the skeleton. With high skeletal uptake no significant activity was to be found elsewhere.

The satisfactory results of $^{90}$Y–MDP organ distribution in healthy test animals (Table 2) were obtained 24 h after iv application: high skeletal uptake. but also a significant activity in liver and spleen.
The more favorable organ distribution results were obtained for $^{90}\text{Y–DPD}$ (Table 2): the uptake in bone was 11–13% ID/g after 24 e.g. 1 h. With high skeletal uptake, low uptake in soft tissues and rapid blood clearance the $^{90}\text{Y–DPD}$ complex proved to be an excellent candidate for tumor therapy as well as in bone palliation.

*The molecular modeling studies:* Thermodynamic stability of radiopharmaceuticals is of the crucial interest, because the loss of radiometal may result in accumulation of radioactivity in non-target organs. Therefore, the selected chelators have to form a complex with high thermodynamic stability to retain its chemical integrity in competition with natural chelators present in a blood stream. Chemical stability of compounds may be judged according to molecular energy data. So, application of molecular modeling as a computation tool is useful for preliminary theoretical analysis of chemical compounds without resorting to laboratory testing. The molecular modeling studies have been carried out for structural analysis of the $^{90}\text{Y–HEDP}$, $^{90}\text{Y–MDP}$ and $^{90}\text{Y–DPD}$ complexes using software HyperChem TM release 6.03 version for Windows.

Conclusion: The experimental results have shown that $^{90}\text{Y-complexes}$ of different polyphosphonate ligands MDP, DPD and HEDP were obtained in high radiochemical purity and with favorable organ uptake, so they may have potential for use in the palliative treatment of bone metastases. Since one of objects was to explore the potential of the therapeutic analogue of $^{99}\text{m-Tc}$-complexion of polyphosphonate ligands MDP, DPD and HEDP, the above developments and encouraging findings with $^{90}\text{Y-MDP}$, $^{90}\text{Y-DPD}$ and $^{90}\text{Y-HEDP}$ gave an impetus to undertake the present study.

2) $^{90}\text{Y complexes of DMSA}$

The object of the study was also labelling of meso-dimercapto succinic acid (DMSA) with Y-90. In this way we try to obtain a labelled compound for therapy of bone malignancies, as
well as for palliative of bone pain. The labelling with $^{90}$Y was carried out by varying experimental parameters such as ligand concentration, pH, time and temperature of the reaction, in order to maximize the labelling yield. Analysis of the complexes enclosed the radiochemical quality control (ITLC, paper chromatography and HPLC), determination of pharmacokinetical parameters and serum stability, as well as organ distribution study in health male Wistar rats. A series UV absorption spectra of yttrium (0.50 mM) solution with increasing meso-DMSA concentration were done too.

HPLC radiochromatograms of labelling mixture showed good separation of $^{90}$Y-DMSA from free $^{90}$Y: R/T was 5.629 min and 6.552 min respectively. The stability of the $^{90}$Y-DMSA complexes was studied at various time points. After preparing (without and with ascorbic acid, with adjusted the pH to 8.0) the $^{90}$Y-DMSA was incubated for 24 h at room temperature and radiochemical purity was analised. $^{90}$Y-DMSA with ascorbic acid as radiolysis stabilizer was found to retain radiochemical purity at as high as 95 % at 24-h incubation. The serum stability of $^{90}$Y–DMSA was assessed by measuring the release of $^{90}$Y from the complex at 37 0C over a 10-day period. The serum stability results for $^{90}$Y–DMSA at different time points showed that the complex prepared with ascorbic acid was quite stable at the studied conditions up to 10 days. No significant dissociation of activity from the complex was observed (the percentage of $^{90}$Y released from this complex at 10 days was <5.0%).

The potential structure of $^{90}$Y-DMSA was proposed according the molecular modelling results. The molecular modeling studies were carried out for structural analysis of the complexes using software HyperChemTM release 6.03 version for Windows.

Our spectrophotometric investigations (unpublished data) suggest that the complex formation between $^{90}$Y$^{3+}$ at very low concentration (10$^{-7}$ to 10$^{-9}$ mol dm$^{-3}$) and DMSA in great excess, depending of the pH value, favoured complexes with metal to ligand mole ratio 1:2. The tetradentate ligand meso-DMSA has been defined as H$_4$L in protonated form with four protonation sites (two sulfur’s atoms in the thiolate moiety and two oxygen’s atoms in the carboxylic acid groups). The proposed conformations of meso-DMSA in solution upon increasing pH value are defined as: H$_2$L$^2$- (I), HL$^3$- (II) and L$^4$+ (the linear structure III). The proposed structure for $^{90}$Y-DMSA complex predicted $[^{90}$Y(LH)$_2$(H$_2$O)$_2$]- complex, which include two six-membered chelate rings via sulfur and oxygen atoms from meso-DMSA. Upon the addition of the second OH$^-$ equivalent to HL$^3$-, another hydrogen bond in internal six-member ring is broken and L$^4$+ species are predominant in solution. The representative and energetically favourable conformation $[^{90}$Y(LH)$_2$(H$_2$O)$_2$]$^{3-}$, which include two five-member chelate rings via sulfur atoms (Fig.5 and Fig 6).

![Fig.5.](image1.png)  ![Fig.6.](image2.png)
Complete results of radiochemical purity control as well as organ distribution study confirmed that $^{90}$Y-DMSA could be obtained with high radiolabelling yield, with high radiochemical purity and with satisfactory organ distribution study. Therefore, $^{90}$Y-DMSA could be also a candidate radiopharmaceuticals for tumour therapy an the palliative treatment of bone metastases.

2. $^{90}$Y-particulates

1) $^{90}$Y-colloids for hepatocellular carcinoma: $^{90}$Y-Sb$_2$S$_3$ and $^{90}$Y-Sn colloid

Radiocolloids as diagnostic and therapeutic agents play an important role in nuclear medicine. The properties of a radiocolloid dispersion, characterized by particle size, shape, charge and stability, are very significant parameters that determine its organ distribution in vivo. In our recent study we try to explore the factors influencing the labeling yield and particle size distribution of $^{90}$Y-labeled antimony trisulfide and tin colloid. Photon correlation spectroscopy (PCS), transmission electron microscopy (TEM) and filtration analysis were used to determine the particle size distribution. The labeling efficiency of $^{90}$Y-labeled colloid particles under various conditions was investigated.

The results of our research have shown that both $^{90}$Y-labeled colloids can be prepared in high yields under optimized conditions. Labeling efficiency of $^{90}$Y-labeled antimony trisulfide and tin colloid was >95 and >88%, respectively. Under well-standardized conditions of the preparation, the reproducibility of the particle size and its distribution is very good, within 7-23 and 85-103 nm for $^{90}$Y-antimony trisulfide and $^{90}$Y-tin colloid, respectively. TEM is a desirable size-measuring technique because individual particles can be observed and measured. A good agreement between TEM and filtration analysis data was found for the particles of both $^{90}$Y-labeled colloids.

Preparation of Y-$^{90}$ colloids for radiosynovectomy

Radiosynovectomy or radiosynoviorthesis is a method for treatment of some joint disorders, often of chronic nature and relatively common in any society. One of the most common causes is rheumatoid arthritis. It is a long suffering from pain, deformity and disability. Radiosynovectomy is a type of radiotherapy used to relieve pain and inflammation from rheumatoid arthritis.

Radiosynovectomy involves local intra-articular injection of suitable $\beta$ -emitting radionuclides in the form of radiocolloids or radiolabelled particulates into the affected synovial joints. These $\beta$-emitting radionuclides penetrate only from fraction of a millimeter to a few millimeters and destroy the inflammatory tissue and thus reduce swelling and pain.

The use of radioparticles for radiosynovectomy are characterised by the radionuclide and the the particulate carrier. The ideal radionuclide is pure $\beta$ -emitter having a short half-life, or $\beta$ -emitter with minimal $\gamma$- emissions, which has a range of between 2 and 10 $\mu$m with low cost, high chemical purity and non-toxic. An ideal particulate carrier must be taken up by the synovial tissue and form a stable complex with radionuclide. It has to be prepared easily and reproducibly, and be non-toxic and non-allergenic. Many radionuclides, namely, $^{166}$Ho, $^{153}$Sm, $^{90}$Y, $^{32}$P, $^{198}$Au, $^{186}$Re, etc. have been identified as potential radionuclides for radiation synovectomy in various particulate forms. Some new radiopharmaceuticals could also be used for radiosynovectomy.
Preparation of $^{90}$Y-HAp

Introduction

In our researches hydroxyapatite (HAp), a natural constituent of bone, was studied as a particulate carrier for beta-emitting radionuclides in radiation synovectomy. Particles were radiolabelled with $^{90}$Y and their in vivo safety was studied following intra-articular injection into knees of normal rats. The aim of our researches was to explore the factors influencing the labelling yield and particle size distribution of $^{90}$Y-labelled calcium hydroxyapatite particles (HAp). We examined the influence of different phosphonates like diphosphonates 1-hydroxy ethyldiene-1,1-diphosphonate (HEDP) as chelator in the yttrium-90-labelled calcium hydroxyapatite particles (HAp) too. In the experiments we used HAp with different particle sizes in order to investigate their biological behavior. Organ distribution studies are performed via two different methods of drug administration, intravenous or intra-articular application.

Materials and methods

Micro hydroxyapatite powders were synthesized in Laboratory for radioisotopes. Vinča Institute of Nuclear Sciences. Hydroxyapatite powder is precipitated via wet precipitation method. Aqueous solutions of calcium hydroxide, Ca (OH)$_2$, and ortho-phosphoric acid (H$_3$PO$_4$, 85%), both of analytical grade, were used as reactants for the preparation of HAp particles, with different sizes.

The labelling was carried out by use of $^{90}$Y in form of $^{90}$YCl$_3$ (in 0.05 mol/dm-3 HCl), supplied from Polatom, Poland. $^{90}$Y was added to 5 mg of HAp in 0.5 ml sterile water into a conic glass veal. The reaction was mixed for 60 min at 37 ºC. The final suspension for injection was made in sterile saline with pH=7.0. Radiolabelling yield was determined by centrifugation (3500 rpm for 5 min) and supernatant was cheerfully separated and radioactivity was measured in both supernatant (free $^{90}$Y) and pellet (particles of HAp labelled with $^{90}$Y). Radiochemical purity was determined by paper chromatography with ITLC-SG strips in 80% methanol and saline. Particle size was analyzed in undiluted samples, at 20ºC, using a light-scattering photon correlation spectroscopy (PCS) instrument Zetasizer Nano ZS (Malvern Instruments Ltd, England, U.K.), which measure particles in the size range 0.6 nm to 6 μm. The in vitro stability of $^{90}$Y-labelled particles $^{90}$Y-HAp as well as with introducing diphosphonate like HEDP as chelators were studied in saline and in 1% human serum at 37ºC. About 0.3 ml aliquots of $^{90}$YHEDP-HAp or $^{90}$Y-HAp were dispensed in 1ml of saline and human serum. At the end of 24h (48, 96… h), the suspension was vortexed thoroughly and centrifuged at 3500 rpm for 5 min. The supernatant was removed and counted for any leaked out radioactivity from the particles. Organ distribution was studied in Wistar rats under anesthesia, after intravenous or intra-articular injection of $^{90}$Y-labelled particles.

Results and discussion

a) Radiolabelling yield and radiochemical purity

Radiolabelling yields of 97-99 % was achieved in all particles preparations. Radiochemical purity of labelled HAp particles was confirmed with ITLC-SG strips in 80% methanol and saline as mobile phases with $R_f$ $^{90}$YCl$_3$=1, $R_f$ $^{90}$Y-HEDP=0, $R_f$ $^{90}$Y-HEDP-HAp=0, $R_f$ $^{90}$Y-HAp=0 and $R_f$ $^{90}$YCl$_3$=1, $R_f$ $^{90}$YHEDP=1, $R_f$ $^{90}$YHEDP-HAp=0, $R_f$ $^{90}$YHAp=0, respectively. Radiochemical purity was > 99 %.
b) In vitro stability studies

The stability of $^{90}$Y-labelled particles was studied in saline as well as in 1% human serum at 37ºC. About 0.3 ml aliquots of $^{90}$Y-HAp as well as $^{90}$Y-HEDP-HAp were dispensed in 1ml of saline and human serum. At the end of 24h, 48h,...up to 5 days, the suspension was vortexed thoroughly and centrifuged at 2000 rpm for 5 min. The supernatant was removed and counted for any leaked out radioactivity from the particles. The experimental results have shown that the $^{90}$Y-labelled particulates showed excellent in vitro stability, > 98% in both media at 37ºC during 5 days.

c) Particle size analysis

The size distribution of synthesized HAp particles (PCS method), presented at Fig. 1. have shown that 75.2% of particles were with diameter of 1.37 μm and 24.8% were with 5.23 μm (Z-Average 2.73 μm).

![Size Distribution by Intensity](image)

Fig. 1: The size distribution of synthesized HAp particles (PCS method)

d) Biological studies

Organ distribution was studied in rats, after intravenous injection of $^{90}$Y-labelled particles $^{90}$Y-HAp or $^{90}$Y-HEDP-HAp. These results were presented at Fig. 2. a) and b) respectively.
Fig. 2. Organ distribution study of $^{90}$Y-HAp (a) and $^{90}$Y-HEDP-HAp (b) after intravenous application.

The results pointed at the fact that $^{90}$Y-HEDP-HAp was stable in vivo with 1% of leaching $^{90}$Y$^{3+}$ accumulated in bone after 24 h. $^{90}$Y-HAp is less stable with 3.5% of leaching activity in the bone.

Fig. 3. Organ distribution study of $^{90}$Y-HAp after intra-articular application.

Organ distribution in rats was studied also after intra-articular injection of $^{90}$Y-labelled particles $^{90}$Y-HAp as well as $^{90}$Y-HEDP-HAp. The results of intra-articular injected $^{90}$Y-HAp were presented at Fig. 3. The similar organ distribution results were obtained for intra-articular injected $^{90}$Y-HEDP-HAp.
The presented results have shown the dependence of organ distribution of radiolabelled particles on a way of drug administration. After intravenous application of radiolabelled particles, greater uptake of $^{90}$Y-HAp and $^{90}$Y-HEDP-HAp was in liver and then in spleen. The results of organ distribution after intra-articular application of radiolabelled particles in rats confirmed that almost 99.1% of radioactive particles. $^{90}$Y-HEDP-HAp as well as $^{90}$Y-HAp, localized in the synovium for at least 96 h, with no detectable activity in the other organs.

**Conclusion**

The present study has shown that $^{90}$Y-labelled HAp particles could be prepared in high yield as well as with excellent radiochemical purity. HAp particles used for labelling were synthesized and characterized in Laboratory for radioisotopes. The labelled particulates have shown high in vitro stability at 37ºC. Biological studies carried out in Wistar rats confirmed complete retention of intra-articular injected radioactivity within the synovial cavity of normal animals for up to 96 h post-injection. Stability of $^{90}$Y-yttrium-HAp complexes increased with introducing diphosphonate HEDP as chelator.

3. **Development of Sr-90/Y-90 electrochemical generator and QC of Ittrium**

$^{90}$ (90/Y) has many favourable features which recommend its application in radionuclide therapy. It has a half-life (64.1 hr) consistent with the rate of antibody accumulation in tumor and no accompanying gamma-ray radiation in its decay. Beta rays have intermediate energy of 0.9367 MeV ($\beta$-max = 2.3 MeV) and a stable daughter ($^{90}$Zr). The important feature is that yttrium – 90 can be attached for many chelate molecules. Furthermore, the considerable path length in tissues of its $\beta$- particles (r$_{95}$ = 5.9 mm) represents a major advantage in solid tumors. Y-90 exists in secular equilibrium with its parent isotope strontium-90, which is a product of fission reaction. There are many impurities which must be removed and pure yttrium – 90 need to convert in appropriate form to be ready for application in medicine therapy. As the demands for radionuclide purity are so high, the obtaining and refinement of $^{90}$Y are very important steps in radionuclide therapy.

A $^{90}$Sr/$^{90}$Y generator system based on an electrochemical separation technique is in development in cooperation with the group from India (M. Venkatesh, Bhabha Atomic Research Center, Mumbai, India) and by use the protocol for the electrochemical separation process developed under the CRP: Therapeutic Radionuclide Generators: $^{90}$Sr/$^{90}$Y and $^{188}$W/$^{188}$Re (IAEA, Technical Reports Series No. 470).

The object of this work was:

1. preparation of $^{90}$Sr- $^{90}$Y generator with useful activity of 3.7 GBq (100 mCi);
2. electrochemical separation of $^{90}$Y;
3. development of the methods for determination of other chemical and radionuclide impurity;
4. transformation solution of $^{90}$Y in appropriate form.
1) Preparation of $^{90}$Sr-$^{90}$Y generator

The equipment for electrochemical separation was completed during a period March-July 2009. A potentiostat unit Potentiostat/Galvanostat/ZRA, Series G 750 (Gamry Instruments, inc) was obtained together with software license FC 350 (Gamry Instruments, inc).

The electrolysis cell is a three electrode system housed in a glass cells fitted with an acrylic cap, made in laboratory of the Faculty of Technology and Metallurgy, University of Belgrade. Two electrodes, an anode and a cathode, dimensions 10x100mm, sealed in glass holder, are high purity platinum plats electrodes made by The Institute for Mining and Metallurgy Bor, Serbia. As a reference electrode saturated calomel electrode Gamry Instruments, inc. was used (Fig. 1). The anode and cathode are fully immersed in solution facing each other. High purity argon gas (from local supplier) was passing through a glass tube, which dipped into the electrolysis solution.

![Fig. 1. The equipment for electrochemical separation (Laboratory for radioisotopes, Institute “Vinca”)](image)

(a) with supplying of argon gas (b)

![Fig. 2. Stable electrolytic potential at platinum cathode](image)
2) Electrochemical separation of $^{90}$Y

Sr-90/Y-90 electrochemical generator developed in Serbia is based on electrolysis of a mixture of $^{90}$Sr and $^{90}$Y in nitrate form at pH 2-3 at a potential of −2.5 V, with 100-200 mA current. $^{90}$Y deposited from 3 mM HNO$_3$ on the platinum electrode, which is used like a cathode. During the second electrolysis, $^{90}$Y is removed from the platinum electrode. In this step, the cathode from the first electrolysis containing $^{90}$Y is used as anode. Upon electrolysis, $^{90}$Y is leached and is deposited to the fresh cathode which is taken out and by dipping in acetate buffer dissolved in a small volume of acetate solution, to obtained $^{90}$Y-acetate, suitable for labelling. For measuring we used dose calibrator (Capintec CRC 15R, USA) which contains calibration factor. For calibrating $^{90}$Y dose secondary calibration sources will be used.

Fig.3. The electrolytic cell completed in Laboratory for radioisotopes, Institute “Vinča” (a) and electrolytic separation of gas H$_2$ (b).

3) Development of the methods for determination of other chemical and radionuclide impurity

$^{90}$Sr breakthrough is the major problem often encountered with $^{90}$Sr/$^{90}$Y generator. Because $^{90}$Sr is a bone seeker, the upper limit of $^{90}$Sr in $^{90}$Y solution for human use is 74 kBq (2 mCi). In order to provide data concerning $^{90}$Sr contamination, development of the methods for determination of chemical and radionuclide impurity was necessary.

The radionuclidian purity of the $^{90}$Y solution was analyzed by paper and ITLC chromatography. Chromatography paper Whatman N° 1 (18 x 2 cm) and ITLC SG strips (14 x 1 cm) and 0.9% saline solution was used for the analyses. During the chromatography, $^{90}$Sr moved with the solvent front, while $^{90}$Y stayed at the origin.

In order to determine the radionuclidian purity of the $^{90}$Y solution we also involved in our experiments so cold “BARC technique”. This method as a combination of solvent extraction and paper chromatography (extraction paper chromatography EPC) was suggested as a sensitive and accurate analytical technique for estimation of the purity of $^{90}$Y. Whatman N° 1 (18 x 2 cm) paper chromatography strip impregnated with 2-ethyl hexyl phosphonic acid...
(KSM-17) at the point of spotting is used. Upon development with normal saline $^{90}\text{Sr}$ moves to the solvent front leaving $^{90}\text{Y}$ completely chelated and retained at the point of spotting. As in the first experiments the mixture of $^{90}\text{Sr}$ and $^{90}\text{Y}$ with low activity, at the megabecquerel level, was used, the activity at the solvent front was estimated by use of dose calibrator (Capintec CRC 15R, USA) which contains calibration factor and compared with the total spotted activity, Fig.4.

In our first experiments the mixture of $^{90}\text{Sr}$ and $^{90}\text{Y}$ with low activity (only few mCi) was used. Therefore this work involved setting up the facilities, standardization of preparing protocol and improving existing quality control (QC) procedures in order to supply reliable products to the national nuclear medicine community. These preliminary results are the confirmation that we successfully completed the equipment for preparing of Sr-90/Y-90 electrochemical generator, established the electrochemical separation technique as well as QC of $^{90}\text{Y}$. In next step, after suppling of some quantities of $^{90}\text{Sr}$ (200 mCi), we could involve production of $^{90}\text{Sr}/^{90}\text{Y}$ generator in order to supply this product to the national nuclear medicine community in Serbia.

Papers


Conferences


Abstract

The aim of this project is to develop a technique for preparation of $^{90}$Sr-$^{90}$Y generator, we have developed a separation technique for isolation of $^{90}$Y from $^{90}$Sr based on using Sr – Spec resin packed in three columns for separation and purification of $^{90}$Sr-$^{90}$Y. The resulting $^{90}$Y is used for therapeutic applications.

The first part of this project described a prototype design for the $^{90}$Sr- $^{90}$Y generator in order to get a very accurate method to obtain the minimum possible $^{90}$Sr Breakthrough. 25 mci of $^{90}$Sr was used in the generator $^{90}$Sr-$^{90}$Y and we obtained the elution yield of $^{90}$Y higher than 88%. Also the eluate was used for preparation of several $^{90}$Y radiopharmaceuticals such as $^{90}$Y-EDTMP and $^{90}$Y-DOTA-HR3. And the work is continues to investigate more radiopharmaceuticals applications in the second part of this project such as $^{90}$Y– FHMA.

In this part of the co-ordination research programmer, A protocol based on results of this studies was developed to prepare and operate a higher activity generator (50-100mci), the resulting elution yield was approximately 94% of $^{90}$Y. The elute was used in preparation of new $^{90}$Y radiopharmaceuticals.

The monoclonal antibodies is still evolving by conjugate Rituximab to The macrocyclic bifunctional chelating agent,(p-SCN-Bn-DOTA)S-2-(4-Isothiocyanatobenzyle)-1,4,7,10-tetraazacyclododecane-tetraaceticacid to obtain the immunoconjugate DOTA-Rituximub in simple way and then investigating the radio labeling conditions with $^{90}$Y.

Experimental and Methods

Generator Preparation and characterization:

The behaviors of $^{90}$Sr and $^{90}$Y on Sr-Spec resin were investigated in different solutions of nitric acids. The results were in good agreement with published results where the highest distribution coefficient of $^{90}$Sr was found at nitric acid concentration of about 3M and at dramatically decreased with diluting nitric acid, while at this concentration the distribution coefficient of $^{90}$Y was minimal. The 3M nitric acid solution was used to separate $^{90}$Y from $^{90}$Sr where Yttrium is eluted while strontium is fixed on the top of the column.

Due to technical considerations assuring the characteristics of the products such as radionuclide purity, the final volume and simplicity of the system, the work was considered three column design connected in series (21x0.4 cm). Each column was filled with one gram of resin and conditioned under 3 M nitric acid solutions.

During separation the $^{90}$Sr was retained on the top of first column, while the other two columns were acted as purifiers of Yttrium, where breakthrough $^{90}$Sr was captured.
Stability of columns:

Investigation of generator repeatability was carried out, where the generator system was loaded with 50 -100mci of $^{90}$Sr and the system was eluted daily with 3M nitric acid (20ml). The results showed that the system was stable for one week (4 elution processes), then after this time the $^{90}$Sr break through was increased by magnitude of $100(^{90}Y/^{90}Sr \geq 10^{-5})$.

While separation of $^{90}$Sr, after elution of $^{90}$Y and cleaning of the columns with distilled water and reusing it, showed the possibility of using the resin for many times.(about 6)

FIG 1 shows a column arrangement for the isolation of $^{90}$Y from $^{90}$Sr stock solutions

Chemical purity of the eluate:

The $^{90}$Y elution was analyzed by using ICP-ms for determination of chemical purity. And the work is continues to investigate more radio pharmaceuticals.

<table>
<thead>
<tr>
<th>Element</th>
<th>Concentration</th>
<th>PPb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td>Ni</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Zr</td>
<td>370</td>
<td></td>
</tr>
<tr>
<td>Sr</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>Y</td>
<td>1x10^-2</td>
<td></td>
</tr>
<tr>
<td>RE*</td>
<td>&lt;1x10^-2</td>
<td></td>
</tr>
</tbody>
</table>
The progress in this stage is the development of preparation and optimization of $^{90}$Sr/$^{90}$Y generator using Sr–Spec resin packed in three columns and purification of $^{90}$Y product.

- Generator was prepared, investigated and validated for different parameters such yield, breakthrough, and it’s usable by any radiopharmaceutical products.

- The design consist of three columns connected in series (long 21 cm, 4 mm diameter, 1 g Sr-Spec) for each one. The first column is used for $^{90}$Sr adsorbent while the second and third are used as safety columns for further purification.

- The stock solution in 3 M nitric acid passing through the columns, $^{90}$Sr is retained, while any yttrium is eluted, then $^{90}$Sr is stripped from the column with dilute 0.05 M nitric acid.

For purification we used AG 50 resin to remove any traces of organic materials and trace element present. The radiochemical of $^{90}$Y product is determined by a combination of the decontamination factor from $^{90}$Sr achieved with the series of Sr–spec columns and that obtained with the purification column (AG 50W X12). A Decontamination factor is the ratio of the concentration of the $^{90}$Sr in the feed stock introduced to the column to the concentration of the $^{90}$Sr in the fraction of interest. The measured was obtained around 3.109.

The solution which contains $^{90}$Y was evaporated then 5 mCi was taken from it for quality control using Sr–spec column. The breakthrough of $^{90}$Sr was more than $10^{-7}$.

The activity of $^{90}$Sr/$^{90}$Y which was used for generator around 50 mCi and the yield was approximately 45 mCi of $^{90}$Y it means more 92% from stock solution.

This figure shows elution behavior using sr-spec several times.
Quality control

Several chromatographic solutions had been used and the best method was to separate the relative amounts of $^{90}\text{Sr}$ and $^{90}\text{Y}$ in the final product by paper chromatography where both of $^{90}\text{Sr}$ and $^{90}\text{Y}$ used are in the form of acetates. Paper chromatography separation was carried out using saline as the mobile phase, on paper W1 spotted with a small amount of DEHPA. After dryness, the paper was cut in 1 cm and counted by LSC.

Chromatographic paper (W1 paper) in the saline of $^{90}\text{Sr}$-$^{90}\text{Y}$ with (50 mCi) activity in stock solution

RCP = 99.56%
Chromatographic diagram using w1 paper in the salin of of $^{89}$Sr/$^{90}$Y with (25mci) activity in stock solution  RCP=99.5%

Breakthrough was $1.10757 \times 10^{-8}$ of Taste 10 after 1 month of $^{89}$Sr/$^{90}$Y with activity (50 mci) in stock solution
Chromatographic diagram for stock solution Whatman w1

(25mci) activity in stock solution

One column chromatography separation of γ-90 used 3MHNO3 and the breakthrough was $\text{Sr}^{90} / \gamma^{90} \leq 10^{-5}$
Malignant cancer is one of the most important resulting in human death. Bone metastases in nearly 25% of all cancer patients; So It is useful to develop radiopharmaceuticals for the treatment of bone cancer.

\[ \text{Sr}^{90}/\text{Y}^{90} \leq 10^{-7} \]

**Study on preparation of \textsuperscript{90}Y-EDTMP:**

Malignant cancer is one of the most important resulting in human death. Bone metastases in nearly 25% of all cancer patients; So It is useful to develop radiopharmaceuticals for the treatment of bone cancer.

\[ \text{Sr}^{90}/\text{Y}^{90} \leq 10^{-7} \]

\text*{90}Y-EDTMP is systemic agent, the injection dose of patients is higher than other diagnostic, it is very important to determination the chemical purity radioactive radio chemical purity of \text*{90}Y-EDTMP,
Experimental methods and result:

Preparation of \(^{90}\text{Y}\)-EDTMP:

\(^{90}\text{Y}\) labeled EDTMP was prepared by dissolving 150 mg of EDTMP in 25% solution of NH4OH and dilution to 10 ml by water, pH was then adjusted to about 8-8.5

The PH = 8-8.5, 1ml of the final solution was labeled with 5 mCi of \(^{90}\text{Y}\), and pH was adjusted to 6.5 by using 0.1 M of ammonium chloride. The formulation was left for 15 min

Purity determination:

Take the labeled solution and measured it on LS(6500) Beckman

St= 100 ũl of \(^{90}\text{Y}\)-EDTMP + 10 ml (0.1M)HCl

Take 100 ũl from solution 15 ml of 0.1M HCl add 100 ũl from solution to column exchange and wash with 0.9 % NaCl

Take 100 ũl from the solution from the column exchange then add 15ml (0.1M) HCl , prepare vile put 15ml (0.1M) inside it as St

Free \(^{90}\text{Y}\) stay in the column exchange

Activity in column Exchange

\[ \text{RCP}\% = \left( 1 - \frac{\text{Initial activity}}{\text{St}} \right) \]

Quality control:

The labeled EDTMP was separated on Whatman paper 3mm with Solution of NH4OH : MeOH: H2O , 0.2:2:4 for 0.5 hr

It was found that RCP \(\geq 98\%\)

Electrophoresis separation on Whatman paper 3mm (21*2Cm)In solution phosphate buffer (0.025M) Na2HpO4 at 210V for 1 hr
The result displayed that the $^{90}$Y immigrated to the cathode and the ($^{90}$Y product) immigrated to the anode as the line analysis shown. RCP was found to be about 98%

We repeated the experiment many times and we found the same results.

**DOTA-HR$^3$ coupling and labeling with $^{90}$Y:**

**Materials and method:**

The result of the immunoconjugate DOTA-HR$^3$ was successfully labeled with $^{90}$Y. The macrocyclic bifunctional chelating agent, (p-SCN-Bn-DOTA)S-2-(4-Isothiocyanatobenzyle)-1,4,7,10-tetraazacyclododecane-tetraaceticacid

DOTA-HR$^3$ was prepared by the addition of 800µl of HR3 pharmaceutical solution (5mg/ml in phosphate buffer, PH=8) to a glass tube percolated with DOTA-SCN (5-5.6mg) in phosphate buffer (0.1M) (PH=8) at room temperature with continuous mild stirring then keep in the fridge.

The DOTA-HR$^3$ coupling during sephadex G50 column activation with (0.1M NH4OAC) were collected. radio labeling was performed at 42°C, the Y-90 (Yttrium III – chloride) solution to be used should have an activity concentration of 2.5mci /ml and where the commercial acquired should be evaporated at 60°C (the became Volume 2.5mci/0.5ml)

**Labeling**

500 µl of this solution $^{90}$YCl (2.5mci/0.5ml) is incubated in (150 µl) Ammonium acetate (0.25M) and 150 µl Ammonium acetate(2M) (Buffer PH=7.5 µl The solution mixed for 10 min then 200 µl DOTA-HR$^3$ was added and stirred for 15 min bringing the total volume up 1ml.
**Quality control:**

Quality control of radio pharmaceutical is carried out by high performance liquid chromatography column: (2.1*200mm) Hypersil AA-ODS – 5micron, Flow: 0.4m/min , λ=254nm Mobile phase : 95%NaCl,5%ACN)-20min ..

The analysis (HPLC) showed an overall radiochemical purity of RCP ≥ 95-98% , The final results are attachment by report.

We repeated the experiment many time and we found the same results

*In this diagram we can see the the immunoconjugate DOTA-HR was successfully labeled with $^{90}$Y at retention time 4min*
In this diagram we can see the $^{90}\text{Y}$ come in rotation time 3min.

This is correspond between the two diagram of free $^{90}\text{Y}$ and the conjugate DOTA-HR3-$^{90}\text{Y}$.
FHMA labeling with $^{90}$Y:
Experimental methods and result:
Preparation of $^{90}$Y-FHMA:

Colloid of ferric hydroxide macroaggregates FHMA was also prepared, where most particle size ranged between 2-10µm in diameter and radiochemical purity was more than ≥99%

The starting (uncoated) super paramagnetic iron nano particles were obtained by mixing aqueous FeCl3 with aqueous NH4OH under sonication at laboratory temperature, for 2min. Then a solution of FeCl2 was added and the mixture was poured into an aqueous of NH4OH, the resulting magnetite precipitate was left aging for 15min and repeatedly washed (7-10 times) with deionizer water. Sodium citrate solution was added under sonication and magnetite was oxidized by slow addition of 5% aqueous solution of sodium hypochlorite, the above procedure of repeated washing afforded the starting primary colloid.

The labeling $^{90}$Y-FHMA was performed by co-precipitation of yttrium and ferric hydroxides under alkaline conditions, as has been done by earlier workers for other metal ions, in brief, to 1 ml $^{90}$YCl3 (74-185MBq or 25 mCi) in 10 ml vial, 4.0 ml of sodium citrate 0.1 M was added, to the previous formed particulates in the room temperature solution was stirred vigorously under sonication then after that it centrifuged, sodium citrate 0.1 M was added with 0.5gr of gelatine in 5ml of saline in boiling water bath and the solution was stirred vigorously under sonication, 1ml of sodium hypochlorite 5% was added. The mixture was centrifuged at 3500 rpm for 5 min the supernatant was removed and counted (almost showed no activity). The percentage yield was calculated as the percent radioactivity associate with the radio labeled particles.

Particle size determination

Particle size measurement were carried out in 2 ml of saline solution added to 1 ml of the particles placed in a 10 ml vial then shaked at 37 and the particles size passed through different size of filters (0.2 -0.8 -2-3-5-10)µm and each fraction was counted

Preliminary estimates of the particle size were accomplished with an optical microscope and scanning electron microscope and the most size of particles about 90% was between 2-10µm.
Particle size between 2-10µm in diameter

Quality control:
Immigration Solution: saline
Whatman paper 3mm
Time of immigration of solution: 1/2hr
Radiochemical purity Y90-FHMA with out gelatin was: 94.45%
Radiochemical purity Y90-FHMA with gelatin was: 99.2%

Radiochemical purity $^{90}$Y-FHMA with gelatin was: 99.2%
Radiochemical purity $^{90}$Y-FHMA without gelatin was: 94.45%.

Radiochemical purity of $^{90}$Y was: 99.8% with salian solution and 3MM W.

This photo shows fraction size 2 µm on optical microscope.
This photo shows fraction size 5 µm on optical microscope

This photo shows Y90-FHMA before filtration on optical microscope
PREPARATION, QUALITY CONTROL STUDIES OF $[^{90}\text{Y}]-\text{DOTA-RITUXIMAB IMMUNOCONJUGATES}$:

Abstract:

Despite the many challenges the use of antibodies in radioimmunotherapy (mAbs) with radioactive metals for cancer diagnosis and therapy has usually been accomplished by the use binifì bifunctional chelating agent. S-2-(4-Isothiocyanatobenzyle)-1,4,7,10-tetraazacyclododecane-tetraaceticacid(DOTA) is one of the most unique compounds for labeling peptides and proteins with multivalent metals suh as yttrium and lanthanides.

Conjugation of rituximab with DOTA-NCS:

(Rituximab) was successively labeled with $[^{90}\text{Y}]-$yttrium chloride after conjugation with macrocyclic bifunctional chelating agent,(p-SCN-Bn-DOTA)S-2-(4-Isothiocyanatobenzyle)-1,4,7,10-tetraazacyclododecane-tetraaceticacid to obtain the immunoconjugate DOTA-Rituximub, Conjugated-Rituximab was obtained by the addition of 100μlof a rituximab pharmaceutical solution (5 mg/ml, in phosphate buffer, pH=7.6) to a glass tube pre-coated with DOTA-NCS at 25°C continuous mild stirring then keep in the fridge.

**radiolabeling of rituximab with $[^{90}\text{Y}]$:**

The conjugated DOTA-rituximab fractions containing the maximum protein content were labeled with $[^{90}\text{Y}]-\text{YCl}_3$ solution. The samples were checked to find the best time scale for labeling. After an hour, the immunoconjun cate DOTA-rituximub was successfully labeled with$[^{90}\text{Y}].$ Radiolabeling was performed at 41-42°C in 1hr DOTA-conjugate, HPLC showed an overall radiochemical purity of 97.5%
This diagram shows the radiochemical purity RCP with Methanol:ammonia 3:2 Using paper Chromatography ITLC-SC The QC of DOTA –NSC-Rituximab $^{90}$Y was 98.1%

This diagram shows the radiochemical purity RCP with Methanol:ammonium acetate 1:1 using ITLC-SC The QC of DOTA –NSC-Rituximab $^{90}$Y was 98.1%
This is correspond between the two diagram the conjugate DOTA-Rituximab of and the conjugate DOTA-Rituximab-$^{90}$Y

References


8. IAEA final report of a co-ordinate research project. Optimization of production and quality control of therapeutic radionuclides and radiopharmaceuticals.
INTRODUCTION

The research project has been conducted at Thailand Institute of Nuclear Technology in accordance to the 1st RCM plan during the IAEA meeting in Warsaw. The objectives of the project include the following 5 specific aims:

1. Development of Sr-90/Y-90 ion-exchange chromatography generator
2. Development of Sr-90/Y-90 extraction chromatography generator
3. Development of quality control technique
4. Development of therapeutic radiopharmaceuticals Y-90 particulates/colloids
5. Development of Re-188 DMSA–bis-phosphonates

Currently we have achieved specific aims 1 to 3. The specific aims 4 and 5 are during investigation. For specific aim 4, we are during the process to extract high purity $^{90}$Y from $^{90}$Sr/$^{90}$Y generator that will yield the starting $^{90}$Y for the production of Y-90 particulates and colloids. For the 5th specific aim, we are on hold to receive the starting agent, bis-Phosphonates, from Dr. Blower group. Therefore, this progress report will cover our work focusing on specific aims 1 to 3.

MATERIALS AND METHODS

For chromatography, Dowex 50Wx 8 (100-200 mesh, hydrogen form, cation exchange resin) was purchased from Sigma, AG 50W-X8 (100-200 mesh, hydrogen form, cation exchange resin) was purchased from Bio-Rad and Ei Chrom resin (SR and RE type) was purchased from EiChrom Technology, LLC. For the tracer, the Sr-85 tracer was purchased from Perkin Elmer. For the starting Y-90 used in the experiment, some Y-90 was purchased from CIS and some was produced in our lab.

1. Development of Sr-90/Y-90 ion-exchange chromatography generator

1.1 Elution profile and optimization of generator system

Dowex 50Wx 8 resin was chosen as absorbing phase and was converted into its sodium form by washing with 1N NaOH and then the free excess base was washed with distilled water. The glass columns (5mm diameter × 200 mm height) were specially made with small bottom end and small side arm tubing near the top end for tight connection to silicon tubing (1/8” ID). Prior to loading the column, the resin was equilibrated with 0.006 M EDTA pH 4.5 for 20 min until the pH of the effluent was unchanged. The flow rate was set at 0.2 ml/min or 0.5 ml/min by peristaltic pump. The fractions were collected for ICP-AES or radioactivity measurement when radiotracer was added. The generator was performed in 3 systems, one column, two
columns and three columns in tandem. Each system was loaded with the combination of inactive Sr and inactive Y at equivalent amount to 1 Ci Sr-90 and 1 Ci Y-90, respectively. The eluents for Y separation were 0.003 M or 0.006 M EDTA at pH of 4.5. In selected systems, Sr-85 (~10 μCi) or Y-90 (~100 μCi) tracer was loaded with the sample to monitor the column performance.

1.2 Recycle of Sr

After Y-separation, the residue Sr which still adsorbed in the column must be eluted out and re-combine to the stock Sr solution. The 0.006 M EDTA at pH 11 and 2 N HNO₃ + 4 N HCl were tried by our group.

Samples of each collected fraction (fixed volume) were taken for data analysis of Sr content by ICP or count for radioactivity when Sr-85 tracer was added.

1.3 Purification of Y

Purification technique was done by the method previously described (Strelow et.al.) using cation exchange AG 50W-x 8 resin, 100-200 mesh, hydrogen form (Biorad) packed in glass column with 0.5 mm ID and 200 mm height. The column was washed at fixed flow rate, 0.5 ml/min, using peristaltic pump with the following solutions in series:

- Washing solution: 0.5 M H₂SO₄ (25 ml, 5ml/fr x 5 fractions)
- 2 M HNO₃ (40 ml, 5ml/fr x 8 fractions)
- 2 M HCl (40 ml, 5ml/fr x 8 fractions)
- 4 M HCl (25 ml, 5ml/fr x 5 fractions)

To validate the method, inactive Y (equivalence to 500 mCi Y-90) and either some amount of inactive metal impurity or Sr-85 or Y-90 were added to the Y-samples loaded. The eluted fractions (5ml/hr) were collected and metal concentration or radioactivity were determined to evaluate the decontamination factor (D.F.).

2. Development of Sr-90/Y-90 extraction chromatography generator

The pre-packed columns, 2 ml, 100-250 µm, SR spec and RE spec Ei Chrom resin were used to separation of Sr-90/Y-90 and to purify Y-90 respectively. The separation and purification methods were adopted from the previous work by Horwitz et. al.

2.1 ⁹⁰Sr-⁹⁰Y Separation

Two and three SR columns were connected in tandem. The elution flow rate was set at 0.5 ml/min. Inactive Sr and inactive Y in equivalent to 1 Ci of Sr-90 and 500 mCi of Y-90, were used as loading sample and spiked with small activity of Sr-85 or Y-90 in separate experiment to monitor column performance or generator efficiency. The eluents for the generator were as follows.

<table>
<thead>
<tr>
<th>Eluent for two column system</th>
<th>Eluent for three column system</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 3 M HNO₃ for 60 ml (3ml/fr x 20 fractions)</td>
<td>3 M HNO₃ for 60 ml (3ml/fr x 20 fractions)</td>
</tr>
<tr>
<td>2. 0.05 M HNO₃ for 20 ml (2ml/fr x10 fraction)</td>
<td>0.05 M HNO₃ for 50 ml (2ml/fr x25 fraction)</td>
</tr>
</tbody>
</table>
2.2 Y-90 Purification

One RE column was used for purification of Y-90 product from any Sr-90 or other metal ions contamination. The column was loaded with inactive Y (equivalent to 500 mCi of Y-90), inactive Sr (equivalent to 10 mCi of Sr-90) and spiked with small activity of Sr-85 or Y-90 to monitor column performance. About 40-80 ml of solution of 2.0-3.0 M HNO₃ with a flow rate of 0.5 ml/min was used as eluent. Each fraction was counted for radioactivity of Sr-85 or Y-90, then D.F. and elution yield were calculated.

3. Development of quality control technique

3.1 Separation of Sr and Y

3.1.1 Validation of extraction paper chromatography method

Validation of extraction paper chromatography method as an efficient method to separate the contaminant Sr-90 in Y product was studied. Sr-85 was used as a tracer to validate the technique. In the experiment, di (2-ethylhexyl) phosphoric acid (Merck) was used as a chelating ligand for the separation of Sr-90 and Y-90 as describe in the previously published protocol (U. Pandey et.al.). TLC-scanner (Bio scan) equipped with proportional counter was used to detect position of radioactivity on the chromatography paper. Then Cobra II automatic gamma counter (Packard) was used to identify whether the detected radiation was generated by gamma emitter (Sr-85) or not.

3.1.2 Factors effecting separation of Sr-90

Effect of solvent and concentration of di (2-ethylhexyl) phosphoric acid in dodecane or hexane (0.1 M and 0.3 M), chemical form of Sr and Y samples with Sr-85 tracer in either chloride or acetate forms and types of mobile phases used in chromatography were also studied. About 5 µl of di (2-ethylhexyl) phosphoric acid was pre-spotted on chromatography paper and allow to air dry then 1 µl of sample (Sr-85+ Sr+Y) was spotted on. Then it was developed in either 0.3 M HNO₃, 0.3 M HCl or normal saline. After drying the chromatography paper was cut into 10 pieces and radioactivity of Sr-85 of each strip was measured by Cobra II automatic gamma counter.

3.1.3 Efficiency of di (2-ethylhexyl) phosphoric acid

Efficiency of di (2-ethylhexyl) phosphoric acid in separation of Y-90 was also done by using Sr-85 as a tracer mixed into variety amount of cold Sr and Y compounds. Conditions used for the separation in this experiment were the efficient conditions established in section 3.2. The chromatograph paper was cut into small pieces then each strip was measured for Sr-85 by gamma counter. In addition, samples of Sr-90/Y-90 at equilibrium condition were studied. Measurement of chromatography paper of Sr-90 and Y-90 were done by Bioscan- TLC scanner immediately after the paper was dried then it was cut and each part of them was added with 10 ml of scintillator (Hisafe). The energy spectra were checked by liquid scintillation counter (Wallac 1414 Winspectral) in Y-90 mode.
3.2 Measurement of Sr-90 in Y-90 Product

3.2.1 GM Counter

GM counting system was also studied as an alternative measurement method. The counting efficiencies of GM-tube (Ludlum model 441 GM detector equipped with Ludlum Scaler) for measurement of Sr-90 and Y-90 were determined as following.

- Standard reference Sr-90 sources were counted without any shielding and with different thickness of aluminum sheet.

- The known activity of stock Sr-90/Y-90 at equilibrium state was spotted on small aluminum trays and chromatography paper and allowed to dry. The samples were counted by GM detector with/without any aluminum shield.

- The known activity of stock Sr-90/Y-90 at equilibrium was used as a sample. The Sr-90 and Y-90 were separated by EPC technique and the chromatography paper was cut into 2 parts and each of them was immediately counted without any shielding.

Each set of data was plot between count rate (semi-log scale) and thickness of aluminum sheet (normal scale). Efficiency of GM tube for 2.28 MeV of Y-90 and 0.546 MeV of Sr-90 were determined from the attenuation curve, respectively.

3.2.2 LSC

After measurement by GM detector, the EPC samples of Sr-90 and Y-90 from section 3.4.1 were immediately analyze for amount of Sr-90 and Y-90 by liquid scintillation counter.

RESULTS

1. Development of Sr-90/Y-90 ion-exchange chromatography generator

1.1 Sr-Y Separation

The concentration of Sr and Y in fractions from ICP-AES measurement or radioactivity counting show the characteristic of each optimizing system as shown in table1, table 2, fig.1 and fig.2

<table>
<thead>
<tr>
<th>Generator system</th>
<th>one column 0.2 ml/min</th>
<th>one column 0.5 ml/min</th>
<th>Two columns 0.5 ml/min</th>
<th>Three columns 0.5 ml/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y-fractions</td>
<td>8 ml (4 fr. peak)</td>
<td>8 ml (4 fr. peak)</td>
<td>8 ml (4 fr. peak)</td>
<td>22 ml (11 fr. Peak)</td>
</tr>
<tr>
<td>average %yield Y</td>
<td>102.61% (tracer counting)</td>
<td>93.96% (tracer counting)</td>
<td>81.35% (tracer counting)</td>
<td>89.7% (By ICP)</td>
</tr>
</tbody>
</table>

Table 1. Elution yields of Sr/Y-Generator one, two and three column system, loaded with inactive Sr +Y with either Y-90 or Sr-85 tracer. Elute was 0.006 M EDTA pH 4.5, flow rate 0.5 ml/min.

It was found that the two column generator system with 0.5 ml/min flow rate was the best system to be used for further development. The same results achieved from the experiments performed with 0.006 M EDTA pH 4.5 or 0.003M EDTA pH 4.5. However, the former method (0.003M EDTA pH 4.5) was preferred in order to keep the same EDTA concentration.
as in the next cycle of separation and to minimize the amount of EDTA which will come out with Sr in the stage of Sr-recycle.

Table 2. Elution yields of Sr/Y-Generator two column system, loaded with inactive Sr + Y plus Y-90 or Sr-85 tracer and elute with 0.003M EDTA pH 4.5 flow rate 0.5 ml/ml comparing to previous results of 0.006 M EDTA pH 4.5 as eluent.

<table>
<thead>
<tr>
<th>Parameter evaluated</th>
<th>Volume 0.003 M EDTA pH 4.5 collected</th>
<th>0.006 M EDTA pH 4.5 8-10 ml 4-5 fr. peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>%Y-yield</td>
<td>76.94 - 99.85</td>
<td>97.83 - 99.88</td>
</tr>
<tr>
<td>D.F. for Sr in Y-fr. collected</td>
<td>6.88x10^2-5.09x10^3</td>
<td>5.5x10^2-3.28x10^3</td>
</tr>
</tbody>
</table>

Fig.1. Elution profile of two column system for Y-separation, eluted with 0.003 M EDTA pH 4.5 (tracer Y-90)

**1.2 Recovery of Sr**

From fraction counting of Sr-85 tracer we found that the most efficient eluent system was 2N HNO₃ + 4 N HCl in which 94-99 % Sr was washed out by 4 N HCl fraction while Sr left in the column was less than 0.1%. Figure 2 and table 3 show the characteristic of the system.

Table 3. %yield of Sr recovered by two eluent system; 0.006 M EDTA pH 11 and 2N HNO₃ + 4 N HCl using Sr-85 as tracer

<table>
<thead>
<tr>
<th>Eluent</th>
<th>0.006 M EDTA pH 11</th>
<th>2N HNO₃ + 4 N HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>volume of Sr fractions</td>
<td>30 ml</td>
<td>30 ml</td>
</tr>
<tr>
<td>%yield Sr recovered</td>
<td>76.4% (tracer counting)</td>
<td>94.39-99.49% (tracer counting)</td>
</tr>
</tbody>
</table>
1.3 Purification of Y

In the first stage of our experiment, the samples of washing and Y fractions from AG 50 x 8 purify column were analyzed by ICP for the efficiency of the system in term of decontamination factor (D.F.) as shown in table 4 and fig.3.

![Column (AG 50 x 8) Purification of Y inactive](image)

Fig.3. Elution profile of Y purification by AG 50 x 8 column. Fractions (5 ml each) were collected for analysis of metal contamination by ICP technique.

Table 4 Decontamination factor of inactive metal impurity in Y fraction analyzed by ICP

<table>
<thead>
<tr>
<th>Metal impurity</th>
<th>Sr</th>
<th>Fe</th>
<th>Cd</th>
<th>Co</th>
<th>Ni</th>
</tr>
</thead>
<tbody>
<tr>
<td>D.F. (in Y fraction)</td>
<td>14.15</td>
<td>4.97</td>
<td>8.52</td>
<td>17.98</td>
<td>51.00</td>
</tr>
</tbody>
</table>

(D.F. is define as the ratio of metal concentration put into the column to its residue concentration leaving in the column in Y fraction collected.)

The maximum D.F. of 3.1x10⁶ for Sr in Y-fraction was obtained as shown in Table 5 and Figure 4.

Table 5 Decontamination factor (D.F.) of Y-purification system by AG 50X8 resin using Sr-85 as tracer

<table>
<thead>
<tr>
<th>system efficiency</th>
<th>volumeY-fractions collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>D.F. for Sr (in Y-fractions)</td>
<td>fr. 18-25</td>
</tr>
<tr>
<td></td>
<td>1.36 - 6.01x10²</td>
</tr>
</tbody>
</table>

The one column system was used to purify 16.61 mCi of Y-90 produced in our laboratory and about 14.53 mCi of (87.4%) Y-90 was obtained. The elution profile was as shown in Figure.5.
2. Development of Sr-90/Y-90 extraction chromatography

2.1 $^{90}$Sr-$^{90}$Y Separation and Sr Recovery

Two and three SR column system give significantly different results on D.F. of Sr. Y-yields did not change very much in terms of volume or number of collected fraction peak. However, high Y-volume resulted in low D.F. as see in Table 6 as well as Figure 6 and Figure 7. The Sr –recovery yield from three column system showed satisfy results (94.86-98.98%) as well as relatively higher D.F. than two column system. The average Sr of 1.4% was fixed in all columns.

Fig. 6. Elution profile of Y-separation by SR – resin, two column system using Y-90 as tracer

Fig. 7. Elution profile of Y-separation by SR – resin, three column system using Y-90 as tracer
Table 6 Y yield, decontamination factor for Sr in Y fraction and Sr recovered yield for extraction chromatography SR resin two and three column system.

<table>
<thead>
<tr>
<th>SR Column System</th>
<th>Volume of 3 M HNO₃</th>
<th>Volume of 0.05 M HNO₃</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 ml fr.3-7</td>
<td>24 ml fr.3-10</td>
</tr>
<tr>
<td>Two column</td>
<td>%Y-yield</td>
<td>98.35 %</td>
</tr>
<tr>
<td></td>
<td>D.F. for Sr in Y-fr.</td>
<td>7502</td>
</tr>
<tr>
<td></td>
<td>%yield Sr Recovered</td>
<td>-</td>
</tr>
<tr>
<td>Three column</td>
<td>%Y-yield</td>
<td>92.95 %</td>
</tr>
<tr>
<td></td>
<td>D.F. for Sr in Y-fr.</td>
<td>2586 - 7021</td>
</tr>
<tr>
<td></td>
<td>%yield Sr Recovered</td>
<td>-</td>
</tr>
</tbody>
</table>

2.2 Purify with RE resin

To optimize the purification system of RE resin (one column), the elution was done with different concentration of HNO₃ and the selected peak fractions to get the high D.F for Sr in Y-fraction were collected. Y-fraction collected at selected fraction peak for 2.5M HNO₃ and 3M HNO₃ resulted in better results than 2M HNO₃ respectively as shown in Table7, Figure 8 and Figure 9.

Table 7 Y yield and decontamination factor (D.F.) of Y-purification system by RE resin using Sr-85 and Y-90 as tracer and washing with three difference concentration of HNO₃

<table>
<thead>
<tr>
<th>system efficiency</th>
<th>HNO₃ concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 M</td>
</tr>
<tr>
<td>Y yield Fr. 4-20</td>
<td>99.78 %</td>
</tr>
<tr>
<td>Fr 8-20</td>
<td>81.37%</td>
</tr>
<tr>
<td>D.F. for Sr (in Y-fractions) Fr. 4-20</td>
<td>3.37</td>
</tr>
<tr>
<td>Fr 8-20</td>
<td>31.76</td>
</tr>
</tbody>
</table>
2.3 Sr-90 breakthrough determination

In EPC-Technique using Whatman No.1 paper, one drop of di(2-ethylhexyl) phosphoric acid was applied at the origin of the paper and air-dried. Y-90 sample in acetate form (1 M NH₄OAc) was spotted at the origin and then developed in 0.9% saline to the solvent front. The strips were cut, upper and lower parts were counted with LSC (Wallac 1414 WinSpectral) for Sr-90 and Y-90 activity (Figs. 10 and 11).

The amount of Sr-90 breakthrough in Y-90 produced in our lab by EPC technique was about 2.2-6.1 \times 10^{-3}\%.

3. Development of quality control technique

3.1 Separation of Sr and Y

3.1.1 Validation of EPC method for separation of contaminant Sr-90 in the Y-90 product

To differentiate spectra of gamma radiation and high energy beta, energy Spectra of (1) $^{85}$Sr, (2) $^{90}$Y and (3) $^{85}$Sr + $^{90}$Y in spectra mode of Cobra II Automatic Gamma Counter were observed as see in Fig 12.
Fig. 12. Energy Spectra of (1) $^{85}\text{Sr}$, (2) $^{90}\text{Y}$ and (3) $^{85}\text{Sr} + ^{90}\text{Y}$ in spectra mode of Cobra II Automatic Gamma Counter

It could be seen that for pure gamma emitter as Sr-85, characteristic spectrum was achieved while the high energy beta emitter could not be seen and the energy spectra of the mixed sample showed count rate at each energy as combination of Fig 12 (1) and Fig 12 (2).

3.1.2 Factors effecting separation of Sr

Better chromatographic separation was observed when dodecane and Whatman No.3 were used as the solvent for preparation of ligand and stationary phase. Figure 13 shows the result from chromatographic separation of strontium and yttrium. It can be seen that 5 µl of 0.1 M HEDHP was able to fix 1 mCi of Y-90 and allow strontium move to the solvent front.

Separation of strontium was not completely when saline was used as a mobile solvent for chromatographic separation. Better separation of strontium and yttrium was found when the mobile phase was 0.3 M HNO$_3$ or 0.3 M HCl. Similar results were observed in 12 cm and 8 cm chromatography paper.

Fig. 13. Radiochromatogram of samples developed in saline, 0.3 M HNO$_3$ or 0.3 M HCl.
3.3 Efficiency of di (2-ethylhexyl) phosphoric acid

Figure 14-16 showed beta spectrum of Sr-90+Y-90, Sr-90 and Y-90, respectively. It can be seen from maximum energy of the spectrum in Figure 2 that Y-90 did not move to the upper part of chromatography paper, indicating that 5 µl of 0.1 M HEDHP is able to complex 1 mCi of Y-90.

![Fig. 14. LSC energy spectra of a) Sr-90 + Y-90 by LSC b) Sr-90 (the upper part of EPC) and C) Y-90 (the bottom part of EPC) by LSC](image)

Figure 14 shows beta spectrum of Sr-90+Y-90, Sr-90 and Y-90, respectively. It can be seen from maximum energy of the spectrum in Figure 2 that Y-90 did not move to the upper part of chromatography paper, indicating that 5 µl of 0.1 M HEDHP is able to complex 1 mCi of Y-90.

3.2. Measurement of Sr-90 in Y-90 Product

![Fig. 15. Attenuation curve of Sr-90 and Y-90](image)

The attenuation curve of reference Sr-90 was shown in Figure 15. Count rate of Y-90 and Sr-90 were determined by performing a least square to the linear part of the measured transmitted fraction on a semi-log plot. The attenuation coefficient of the beta-particles, $E_{max}$, (in a unit of cm$^2$ g$^{-1}$) for Al plate of Y-90 and Sr-90 are calculated following the empirical formula as reported by O. Gurler et. al.,

$$\mu = 17 E_{max}^{-1.43}$$  \hspace{1cm} (1)

The transmission rate, T, of beta-particles through some shielding material is approximately described by an exponential equation, viz.
\[ T = \frac{I(t)}{I(0)} = e^{-\lambda t} \]  \hspace{1cm} (2)

Table 8 shows that the amount of Sr-90 and Y-90 in the Sr-90/Y-90 samples (at equilibrium state), after extraction paper chromatography, measured either by LSC or GM detector were almost the same which they theoretically should be for the parent and daughter nuclides at their secularly equilibrium state.

<table>
<thead>
<tr>
<th></th>
<th>Y-90</th>
<th>Sr-90</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM Counting efficiency</td>
<td>0.34</td>
<td>0.245</td>
</tr>
<tr>
<td>Count rate of sample (cpm)</td>
<td>6108</td>
<td>4342</td>
</tr>
<tr>
<td>Activity (Bq)</td>
<td>299</td>
<td>295</td>
</tr>
<tr>
<td>LSC (Bq)</td>
<td>292</td>
<td>295</td>
</tr>
</tbody>
</table>

**CONCLUSION**

Y separation of Sr/Y generator gave high yields (81.35-102.61%) of product in all system. Trace amount of Sr; about <0.7% (0.09-0.63) of total Sr loaded; were founded in 8 ml of Y fraction collected for one and two column system and 26 ml for three column system.

The Sr content can be kept lower by collecting Y at only 3-6 ml high activity peak. The system will be selected based on high Y yield, low Sr contamination and short processing time.

The washing process was failed to recycle Sr back in high yield. Data from Sr-85 tracer measurement showed that the recovery yield was only 76.4% by 0.006 M EDTA pH 11, 54.6% by 4M HCl and 57.3% by 2N HNO₃+4N HCl. The system should be optimized for highest Sr recovery yield to prevent high Sr loose in the next cycle.

Purification of Y by AG 50x 8 gave relatively low D.F. in this experimental model. Further studies are needed to be done such as using higher crosslink resin or changing of eluent concentration or etc.

Quality control of the final product could be done by separation of Sr and Y by EPC technique which 0.1 M HEDHP in dodecane is used as ligand to complex yttrium and amount of contaminated Sr-90 could be measured by LSC. GM counter could be used as an alternative method when the two radionuclides were separated from each other and the counting efficiency of the detector of 2.28 MeV of Y-90 and 0.546 MeV of Sr-90 were done using standard referent Sr-90 source and attenuation curve.

**REFERENCE**


- P.E. Horwitz, L. Mark; Process for the Separation and Purification of Yttrium-90 for Medical Applications.


Bifunctional bisphosphonate complexes with $^{99m}$Tc and $^{188}$Re for the diagnosis and therapy of bone metastases

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1,1-Bisphosphonates (BPs) are a family of compounds extensively used in the management of disorders of bone metabolism. They accumulate in areas of high bone metabolism, such as bone metastases, and consequently have been receiving increasing attention as molecular imaging probes and pain palliation treatments. Imaging bone metastases with BPs using single photon emission computed tomography (SPECT) or planar scintigraphy is one of the most often-performed clinical imaging procedures. Beta-emitting analogues capable of producing a therapeutic effect have also been developed. In particular, the rhenium compounds $^{186/188}$Re-hydroxyethylidene-1,1-diphosphonate (Re-HEDP) have shown promise as palliative agents for bone metastases in recent clinical trials. The radiochemicals consist of a complex of a BP (e.g. methylene diphosphonate, MDP) with gamma- ($^{99m}$Tc) or beta- ($^{186/188}$Re) emitters.

Despite the proven clinical success of $^{99m}$Tc/$^{188}$Re-BPs, these radiopharmaceuticals are far from optimal from a chemical and pharmaceutical point of view. For example, despite decades of clinical use, their structures and compositions remain unknown. A critical review of the literature reveals that the $^{99m}$Tc-MDP preparation used in the clinic is composed of a mixture of anionic polymers of different properties. A particular concern is that the in vivo stability of $^{186/188}$Re-BPs does not adequately match their physical half-life and a large fraction of the injected complex degrades to perrhenate in vivo within 24 h, leading to reduced bone uptake and higher soft tissue doses. Furthermore, $^{186/188}$Re-BPs are not chemically analogous to their Tc-counterparts and do not target bone metastases unless additional “carrier” non-radioactive rhenium is added. Consequently there is a need for rational design of $^{186/188}$Re-labelled BP derivatives to improve specificity and reduce soft-tissue and bone marrow doses during radionuclide therapy.

In current Tc/Re-BP complexes the BP acts as both chelator and targeting group. Each role, however, may compromise the other as BPs are excellent bone-seeking agents but poor Re chelators. To improve upon current $^{186/188}$Re-BPs, a more logical approach is the use of targeted bifunctional ligands in which the targeting (BP) and metal chelating groups are separated within the molecule so that they can each function independently and effectively. A few recent reports describe such a BP-chelator bifunctional approach. These BP-conjugates, however, require complicated multi-step synthetic strategies, show high plasma protein binding and often form enantiomeric mixtures. Herein we report a new chelator-BP conjugate that is synthesised using mild aqueous conditions in one step from commercially available compounds. In addition, efficiently forms single well-defined isostructural Tc/Re complexes with no detectable protein binding that efficiently accumulate in bone tissue in vivo.
Our approach requires the BP part of the molecule to be separated from the chelator by a spacer, to avoid any BP-metal interactions. In addition, the radionuclide must selectively coordinate with the chelating group, not the BP, and must remain inert under in vivo conditions. The organometallic precursor \( \text{fac-}[\text{M(CO)}_3(\text{H}_2\text{O})_3]^+ \) (\( \text{M} = \text{Tc, Re} \)) (Scheme 1B, 4), pioneered by Jaouen et al. and Alberto et al. facilitates the latter requirement. When the three labile water molecules are displaced by an appropriate ligand system, the d6 low-spin octahedral Tc(I)/Re(I) centre formed is protected from oxidation and ligand substitution. Furthermore, imaging probes containing a coordinatively saturated \( \text{fac-}[\text{M(CO)}_3]^+ \) core have shown high in vivo inertness and negligible binding to human serum proteins. Particularly favourable ligands for \( \text{[M(CO)}_3]^+ \) are N3-tridentate chelators containing two \( \text{sp}^2 \) N-heterocycles, such as dipicolylamine (DPA). As the targeting vector we selected alendronate (2) (Scheme 1A), a clinically-approved BP that binds avidly to hydroxyapatite (HA), the main component of bone mineral. Two major obstacles were encountered during the development of DPA-alendronate (3, Scheme 1A). First is the insolubility of alendronate in organic solvents, which complicates conjugation reactions. Second, the high basicity of its amino group (\( \text{pK}_\text{a} \) 12.7) inhibits nucleophilic attack by alendronate using standard organic bases. Important factors to overcome these barriers are pH and the concentration of base. Using strong organic bases such as triethylamine was unsuccessful. High concentrations of inorganic bases and high temperatures, however, led to hydrolysis of 2-picolyl chloride (1, Scheme 1A) and rearrangements of the BP. We found that using water as solvent and maintaining the pH of the solution at 12 with the minimum amount of NaOH was sufficient to drive the reaction to completion after 36 hours at room temperature, without detectable hydrolysis or BP rearrangements. The yield of 3 was >90% by RP-HPLC.

**\(^{99}\text{Tc}\) and \(^{187/185}\text{Re}\) Studies**

The complexation of 3 with fac-[Re(CO)]\(^+\), and its solution properties, were examined using HPLC and NMR, MS and IR spectroscopies (Fig. 1). The aim was to determine whether the organometallic core selectively coordinated the chelating DPA group. NMR/HPLC titration
studies revealed that fac-[Re(CO)_3(H_2O)_3]^+ stoichiometrically binds 3 in the designed facial conformation when less than 1.5 equivalents of fac-[Re(CO)_3(H_2O)_3]^+ were used. The presence of a single species corresponding to 5 in solution was confirmed by $^1$H/$^31$P-NMR and HPLC (Fig. 1, Fig. S1). HR-ESI-MS also demonstrates the formation of the desired product. Upon addition of 1.5 equivalents or more of the metal reagent, new $^31$P-NMR signals appeared, accompanied by a general upfield shift of the aromatic protons in the $^1$H-NMR spectrum strongly suggesting coordination of metal centres to the BP group. These putative multinuclear species, however, do not form during radiosynthesis as the concentration of ligand always exceeds that of the radionuclide by several orders of magnitude. 3 in concentrations as low as $10^{-5}$ M (0.7 µg per labelling) can be efficiently labelled with fac-[$^{99m}$Tc(CO)_3]^+ in water to form 6 (>98% radiochemical yield, 22 GBq/mg). RP-HPLC analyses show that 5 and 6 coeluted, demonstrating their analogous structure (Fig. S2).

Fig. S1. RP-HPLC chromatograms of 3 (top left) and 3 plus increasing amounts of [Re(CO)_3]^+.
One of the factors that makes 2 one of the most potent BP drugs is its high skeletal uptake and retention, which is directly related to its affinity towards HA. The affinities and selectivities of 6 and 99mTc-MDP towards several calcium salts were evaluated using an in vitro assay. As shown in figure 2, 6 binds HA selectively with very high affinity (>80% binding). 99mTc-MDP, on the other hand, is less selective and binds HA and calcium oxalate (CO) with lower affinity (~40% binding). Remarkably, 6 shows higher affinity for HA despite having a concentration of competitive inhibitor (in the form of non-labelled BP) ~10 times higher than in the 99mTc-MDP preparation.

The fate of a targeted imaging probe or radiopharmaceutical in blood is one of the most important factors during pre-clinical development of radiopharmaceuticals. Strong binding to
serum proteins such as albumin often delays blood clearance, leading to low target-to-background ratios.\textsuperscript{11} Previous chelator-BP conjugates have shown high binding to serum proteins.\textsuperscript{9} Furthermore, human plasma enzymes may decompose exogenous compounds. Complex 6 showed negligible binding to serum proteins and no decomposition after incubation with human plasma for at least 18 h (Fig. S4). \textsuperscript{99m}Tc-MDP, on the other hand, remained mostly bound to serum proteins throughout the 18 h incubation.

![Graph showing protein-unbound (%)](image)

**Fig. S4.** Serum protein binding study of 6 (black circles) and \textsuperscript{99m}Tc-MDP (empty circles).

*In vivo* imaging studies with 6 were carried out with adult Balb/C female mice using a nanoSPECT/CT animal scanner. Control imaging studies were also performed with \textsuperscript{99m}Tc-MDP. 6 shows essentially identical bone uptake to \textsuperscript{99m}Tc-MDP, demonstrating its usefulness as a bone-seeking agent (Fig. 3A, Fig. S5). Biodistribution studies were performed ex vivo to quantify the uptake of the two tracers in bone and soft-tissue organs (Fig. 3B). As expected, the bone uptake of both compounds was very high, with 27-30\% of the injected dose per gram of tissue (\% ID/g) in the femur. Imaging shows that this uptake was in fact confined to the joints, where active remodelling occurs. Liver and lower gastrointestinal uptake, while very low, were slightly higher with 6 (2.5\% ID/g) than with \textsuperscript{99m}Tc-MDP (0.4\% ID/g), consistent with the more lipophilic nature of the tricarbonyl core compared to MDP. An advantage of using bifunctional compounds is that properties like lipophilicity may be tuned by using, for example, different spacers and/or chelators.
Fig. 3 (A) SPECT(colour)/CT(greyscale) image showing the high uptake of 6 in bone tissue, particularly at the joints. (B) Biodistribution profile of 6 (black bars) and 99mTc -MDP (grey bars) at t=6.5 h post-injection.

Fig. S5 SPECT/CT images showing the essentially identical bone uptake of 99mTc -MDP (A) and 6 (B) in mice.

188Re Studies

The synthesis of fac-[\(^{188}\text{Re} (\text{CO})_3 (\text{H}_2\text{O})_3\)]\(^+\) is not straightforward and therefore several methods for its formation were investigated:

1. The method of Schibli et al.\(^{17}\) resulted in yields of 85%, consistent with the reported yields. 5 mg BH3·NH3 were placed in 10 mL glass vial. This method is as follows: a
vial was sealed with an aluminum capped rubber stopper and flushed with CO for 20 min. The $^{188}\text{Re}$ generator eluate (as $^{188}\text{ReO}_4^-$) (1 mL, 700 MBq) was mixed with 6 µL of concentrated $\text{H}_3\text{PO}_4$ (98%) prior to the injection in the reaction vial. The vial was then incubated at 60 °C for 15 min. Pressure from the evolving $\text{H}_2$ gas was balanced with a 20 mL syringe. The reaction was cooled on an ice bath. The final pH of the reaction solution was neutral and the purity was assessed by HPLC and TLC.

2. The method of Park et al.$^{18}$ resulted in yields of 60% or lower in our hands. This method is as follows, a 10 mL vial containing tetrahydroborate exchange resin (BER supplied by S. H. Park under this CRP, 3 mg), borane–ammonia (BH$_3$·NH$_3$, 3 mg), and potassium boranocarbonate (K$_2$[H$_3$BCO$_2$], 3 mg) were capped with a rubber stopper. A solution of sodium perrhenate (as $^{188}\text{ReO}_4^-$) (1 mL, 700 MBq) and 7 µL of concentrated H$_3$PO$_4$ (85%) were added into the vial by a 20 mL syringe and then heated to 60 °C in a boiling water bath for 15 min. Pressure from the evolving gas (about 10 mL) was balanced with the syringe and cooled to room temperature.

3. A modification of the method 1 in which the final product is purified to >95% radiochemical purity by passing the solution through an OnGuard-Ag column (Dionex) and a Bondelut SAX (Varian) column connected in series.$^{19}$ Using this method only radiochemical yields of 33% were achieved after purification (radiochemical purity >95%).

4. A combination of methods 1 and 2 in which a 20 mL vial is charged with 3 mg of BH$_3$·NH$_3$, 3 mg of BER and flushed with CO gas.$^{19}$ The $^{188}\text{Re}$ generator eluate (as $^{188}\text{ReO}_4^-$) was passed through OnGuard-Ag column (Dionex) to remove the chloride ions and then mixed with 7 µL of concentrated $\text{H}_3\text{PO}_4$ (85%). Injection of this solution into the vial resulted in the release of $\text{H}_2$ gas that was balanced with the 20 mL syringe used for the injection. The vial was then heated to 60 °C in a boiling water bath for 15 min and cooled to room temperature afterwards. Filtration of the solution using a standard 0.22 µm cellulose filter resulted in the retention of > 60% the activity in the filter. The filtrate, however, was 99% radiochemically pure. Alternative methods of filtration, such as hydrophilic PTFE are currently being evaluated.

3 was labelled with fac-[$^{188}\text{Re}(\text{CO})_3(\text{H}_2\text{O})_3]^+$ obtained using method 3. Briefly, 1 mL of - [$^{188}\text{Re}(\text{CO})_3(\text{H}_2\text{O})_3]$ (170 MBq) in 1 mL were added to a vial that had been previously flushed with N$_2$ gas for 10 min and had been charged with PBS buffer (as a solid, to make a final 1 mL 1xPBS solution). Then 100 µL of a 1.7x10$^{-3}$ M solution of 3 in carbonate buffer were added. The vial was then heated for 30 min at 75 °C. After cooling the solutions in a water bath the sample was analysed by analytical RP-HPLC. $^{188}\text{Re}(\text{CO})_3$-3 was obtained in a radiochemical yields of 95%. The specific activity achieved was 2 GBq/mg, although a more comprehensive study to determine the maximum specific activity achievable remains to be performed. According to the $^{99m}\text{Tc}$ studies (vide supra) there is not reason a priori why specific activities of the ordr of >1 GBq/mg should not be achievable.

**In vivo studies: Comparison of $^{188}\text{Re}(\text{CO})_3$-3 and $^{188}\text{Re}$-HEDP.$^{19}$**

In order to assess the bone uptake and biodistribution of $^{188}\text{Re}(\text{CO})_3$-3 a series of imaging studies were performed. Thus, 33 MBq (200 µL) of $^{188}\text{Re}(\text{CO})_3$-3 were injected in female Balb/c mice (x3) and scans acquired in a nanoSPECT-CT scanner at 1, 5, 24 and 48 h timepoints. The same studies were performed with $^{188}\text{Re}$-HEDP as a control.
As shown in figure 4, $^{188}$Re(CO)$_3$-3 uptake is essentially the same as with 6 (Figs, 3 and S5). Furthermore, the retention of $^{188}$Re(CO)$_3$-3 appears to be higher than with $^{188}$Re-HEDP, consistent with the superior stability and targeting properties of $^{188}$Re(CO)$_3$-3.

Fig. 4. nanoSPECT-CT images at 24 h post injection of $^{188}$Re-HEDP (left) and $^{188}$Re(CO)$_3$-3 (right)

The increased retention and higher stability of $^{188}$Re(CO)$_3$-3 is also evidenced in a (preliminary) biodistribution study at 48 h post injection (Fig 5, top). Thus, $^{188}$Re(CO)$_3$-3 shows a very similar biodistribution pattern to 6, with the majority of activity bound to bone tissue (21% ID/g) and only a small fraction of uptake in the kidneys and liver (~2% ID/g). Interestingly, $^{188}$Re(CO)$_3$-3 shows higher bone uptake than $^{188}$Re-HEDP, as evidenced by the biodistribution study at 48h and the nanoSPECT-CT imaging at 24 and 48h (Fig 4). The stability of $^{188}$Re(CO)$_3$-3 is also demonstrated by the higher thyroid uptake of $^{188}$Re-HEDP (Fig 5, bottom), derived from its decomposition into $^{188}$ReO4.

Fig 5. Left: Preliminary biodistribution study (48 h post injection) of $^{188}$Re-HEDP (black) and $^{188}$Re(CO)$_3$-3 (blue); Right: Expansion of thyroid uptake data
Thus, these results demonstrate that 3 forms well-defined, well-characterised and stable bone-targeting agents with the fac-[M(CO)3]⁺ (M = Tc, Re) metal core and deserves further evaluation for the radiotherapy (188Re) of bone metastases.

Acknowledgements.

We would like to thank Cancer Research UK (Grant C789/A7649) for funding, C. Finucane and S. J Mather for the in vivo studies, Dr S. H. Park for providing the borohydride-exchange resin, Covidien for providing the Isolink™ kits and Dr Jim Ballinger (Nuclear Medicine, Guy’s and St Thomas’ Hospital, London) for supplying [99mTcO₄]Na and for useful discussions.

References


16. The solution of 6 used in these studies contained 10 times more non-labelled BP than in the $^{99m}$Te-MDP solution. To test the inhibition properties of 3', the same binding studies were carried out with HA and CO in the presence of an excess of 3', resulting in the complete inhibition of binding of 6.


Country report: Vietnam

SETTING UP OF A $^{90}$Sr/$^{90}$Y GENERATOR SYSTEM BASED ON SUPPORTED LIQUID MEMBRANE (SLM) TECHNIQUE AND RADIOLABELING OF eluted $^{90}$Y WITH BIOMOLECULES

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In the course of participating in the IAEA-CRP during the last two years, Vietnam has achieved the goal of setting up a $^{90}$Sr/$^{90}$Y generator system based on Supported Liquid Membrane (SLM) technique and also radiolabeling of the eluted $^{90}$Y with antibody, peptides and albumin. A two stage SLM based $^{90}$Sr/$^{90}$Y generator was set up in-house to generate carrier-free $^{90}$Y at different activity levels viz. 5, 20, 50 mCi. The generator system was operated in sequential mode in which 2-ethylhexyl 2-ethylhexyl phosphonic acid (PC88A) based SLM was used in the first stage for the transport $^{90}$Y in 4.0 M nitric acid from source phase where $^{90}$Sr-$^{90}$Y equilibrium mixture is placed in nitric acid medium at pH to 1-2. In the second stage, octyl (phenyl)-N,N-diisobutylcarbamoylmethyl phosphine oxide (CMPO) based SLM was used for the transport of $^{90}$Y selectively to 1.0 M acetic acid which is the best medium for radiolabeling. The eluted $^{90}$Y from the generator was tested for the presence of any traces of $^{90}$Sr using the Extraction Paper Chromatography (EPC) and was found suitable for radiolabeling. The generator system could be upgraded to 100 mCi level successfully due to an expert mission from India through IAEA. The $^{90}$Y product obtained from the generator system was used for radiolabeling of antibody and peptides viz. Rituximab, DOTATATE and albumin particles. A new chromatography system could be developed for analyzing $^{90}$Y labeled albumin using the TAE buffer as mobile phase in PC and ITLC.

DESCRIPTION OF RESEARCH CARRIED OUT

With the aim of setting up a Supported Liquid Membrane (SLM) based $^{90}$Sr/$^{90}$Y generator system, 100 mCi/1 mL of $^{90}$Sr(NO$_3$)$_2$ in 1.5 M HNO$_3$ was received from IAEA in November, 2008 and 2-ethylhexyl 2-ethylhexyl phosphonic acid (PC88A) was bought from Daihachi Chemicals Industry Co. Ltd, Japan. Octyl (phenyl)-N,N-diisobutyl carbamoylmethyl phosphine oxide (CMPO) was bought from Strem Chemicals, Newburyport USA (CAS No.[83242-95-9], Lot No. A4546029). In addition, one Poly methyl methacrylate (PMMA) box and five double glass-cells were fabricated.

Studies were carried out further to develop the $^{90}$Sr/$^{90}$Y generator system based on supported liquid membrane technique. Initially, two stage system was developed at different activity levels viz. 5, 20 and 50 mCi levels and operated in sequential mode. In the first stage, PC88A based SLM was used which transported $^{90}$Y from nitric acid medium containing pH ~1 (first chamber) to 4M HNO$_3$ (second chamber). In second stage, the $^{90}$Y product from first stage was transferred to the first compartment of the second stage using CMPO based SLM where 1M acetic acid was used as receiving phase for $^{90}$Y. The quality controls of the product using extraction paper chromatography and separation yields were evaluated. The $^{90}$Y product obtained from the generator system was used for radiolabeling of antibody and peptides viz. Rituximab, DOTATATE and albumin particles. The experimental results from these studies carried out during last 18 months are presented in this report.
1. Two stage $^{90}$Sr/$^{90}$Y Generator System in Sequential Mode:

*a. $^{90}$Sr-$^{90}$Y generator system at 5, 20, 50 mCi levels*

Four glass cells were connected together with magnetic stirrers as shown in Fig. 1. The solvent impregnated PTFE membranes were incorporated in between the chambers. In the first stage, undiluted PC88A and in second stage 0.8M CMPO in n-dodecane were used as carrier. For solvent impregnation, PTFE supports were kept immersed in the solvent overnight. Before use they were washed with deionized water and tightly assembled into the generator set up. The PC88A based SLM was inserted and tightly fixed in between the first and second chamber (First Stage). In the same way, the CMPO impregnated PTFE was inserted and tightly fixed between the third and the fourth chamber (Second Stage). The generator was housed in a PMMA box inside a fume hood in order to protect and avoid any radioactive contamination. The first chamber was filled in with 5 mL of 0.1 M HNO$_3$ containing $^{90}$Sr,$^{90}$Y activity mixture. The second chamber was filled with 5 mL 4.0 M HNO$_3$; the third chamber was filled in with 5 mL of 4.0 M HNO$_3$ and the fourth chamber was filled with 5 mL of 1.0 M acetic acid. In order to introduce the solution into the chamber or collect the liquid product from the chamber, the Teflon tubes of small dia of ~2 mm were connected between the chambers as shown in the Fig. 2. The solutions in chambers were stirred by using PTFE coated magnetic stirring bars. The generator set up was placed on the magnetic stirring system.

The transport of beta activity was carried out as a function of time. The experiments were carried out at two different activity levels viz. 20 and 50 mCi levels. The activity data were used to evaluate the separation yields. Three set of experiments were carried out under identical experimental conditions. The system was operated continuously for about 5 h in the first stage. Over time, the beta activity due to $^{90}$Y was transported from the first to the second chamber across the PC88A based liquid membrane. In the next step, $^{90}$Y transported in 4 M HNO$_3$ was removed and filled in the third chamber which contained CMPO based SLM and 1 M acid acetic as receiving phase. The second stage was also operated continuously for about 6 h. Finally, the entire product containing $^{90}$Y in acetic acid was taken out for quality control.

![Fig. 1. $^{90}$Sr/$^{90}$Y Generator using Supported Liquid Membrane technology](image)
b. \(^{90}\text{Sr} - {^{90}\text{Y}} \text{ generator system at 100 mCi level}\)

Various lots of \(^{90}\text{Sr}\) (viz. 5 mCi \(\times\) 2, 20 mCi and 50 mCi adjusted to pH 1-2) which were utilized earlier for the separation of carrier-free \(^{90}\text{Y}\) were collected as follows: First two lots of 5 mCi each and one lot of 20 mCi were mixed in glass beaker and evaporated under IR lamp for volume reduction to \(~1-2\) mL. This solution (expected \(^{90}\text{Sr} \sim30\) mCi) and 20 mCi of \(^{90}\text{Sr}\) left over from the original supplied stock were transferred to a glass vial using the solution lot of 50 mCi. This mixed solution was adjusted to pH 1-2 and analysed for \(^{90}\text{Sr}\) by radiochemical method. The solution was found to contain 98.76 mCi of \(^{90}\text{Sr}\) and used as feed for compartment-1.

2. Quality control of \(^{90}\text{Y}\) extracted from \(^{90}\text{Sr}/^{90}\text{Y}\) generator

To evaluate the radionuclidic purity of \(^{90}\text{Y}\) acetate, the Extraction Paper Chromatography (EPC) method was adopted by using PC88A as chelating agent.

**Application of the EPC method:**

Whatman No.1/ITLC paper: 1.0 cm x 12 cm or 1.5 cm x 12 cm  
Developer: Saline solution (0.9% NaCl in water)  
Chelating agent: PC88A at the point of spot.

In these quality control testing, about 10 \(\mu\)L of PC88A was spotted between the second and the third segments of the Whatman No.1/ITLC paper (in a spot, \(d = 1\) cm). After its drying, 5 \(\mu\)L of \(^{90}\text{Y}\) sample was pipetted over the PC88A spot. The paper was developed in saline solution. The paper strips of 1 cm length were cut and subjected to activity counting using GM counter initially. The \(^{90}\text{Sr}\) radioactivity migrated at the solvent front was taken and placed in 10 mL liquid scintillation vials and counted. The measured counts were directly compared to the total introduced activity to obtain the radionuclidic impurity in \(^{90}\text{Y}\).

To evaluate the radiochemical purity of \(^{90}\text{Y}\) acetate, NaCl 0.9% (\(R_f = 0.9-1.0\)) was used. Dosimetric evaluation was carried out in PC and ITLC methods using Tris-acetic-EDTA (\(R_f = 0.9-1.0\)), amoni acetate 10% - methanol 30:70 (\(R_f = 0.0\)), Tris-NaCl-EDTA (\(R_f = 0.9-1.0\)) and 0.1 M sodium acetate (\(R_f = 0.9-1.0\)).
3. Labelling of Rituximab with Y-90

Rituximab was bound to the DTPA chelating agent using Hnatowich methods. Cyclic anhydride DTPA (cDTPAa, 0.1 mg/mL) was dissolved in chloroform and was degassed under a stream of nitrogen for 30 minutes. Rituximab solution in 0.05 M bicarbonate buffer was immediately added and mixed for one minute at room temperature. The antibody Rituximab at different concentration (5 mg/mL and 10 mg/mL) was coupled with the cDTPAa, at molar ratios (cDTPAa : Rituximab) of 1:1, 3:1, 5:1, 10:1 and 20:1. The conjugation of DTPA-Rituximab mixture was labelled with Y-90, then purified using Sephadex G25 in order to determine coupling efficiency.

4. Labelling of DOTATATE with Y-90

DOTATATE was supplied by Dr Chinol (IEO, Italy). For labelling with Y-90, DOTATATE in the form of lyophilization was dissolved in sterile DDW at a concentration of 1 mg/mL. Ytrium-90 obtained from $^{90}$Sr/$^{90}$Y Generator based on SLM in the form of $^{90}$Y-acetate was used for the experiments. Gentisic acid (0.33g sodium acetate, 0.4g of 2, 5 dihydroxybenzoic acid, 250 $\mu$L saturation NaOH and 10 mL sterile DDW, pH 5) was prepared. This study aimed at optimization of labelling conditions for $^{90}$Y-DOTATATE, such as pH, reaction time. The optimized protocol for labelling was performed as following: 10 $\mu$g (10$\mu$L) of DOTATATE was added into 50 $\mu$L gentisic acid and 37 MBq of Y-90-acetate at pH 4.5 – 5. The reaction mixtures were incubated at 90ºC for 30 min, and then quality control procedures were conducted.

5. Labelling of albumin particles with Y-90

In order to label albumin with Y-90, the directed methods were applied in this study. Both in house prepared albumin and MAASOL of Italy were used. The preparation of wet albumin particles with size of 10 - 30 $\mu$m was carried out in our laboratory. The solution of 0.16% HSA, pH 4.6 was suspended in 5% NaCl, pH 6 at 80ºC with stirring. The microaggregated albumin particles size was ranged from 10 $\mu$m to 30 $\mu$m. The suspended albumin particles were centrifuged at 3000 rpm for 5 min. The precipitates were reconstituted with 0.8M sodium dihydrophosphate. The 2 mg of albumin particle and 0.5 mg of stanous chloride dihydrate in 2 M HCl were mixed; adjusted pH 5 with 2 M NaOH. The size of particles was examined with an optical microscope and a hemocytometer. The mixture was washed three times with phosphate buffer saline, pH 7.2 by centrifugation and resuspended in 0.5M sodium acetate buffer, pH 6. Yttrium-90 in 1.0 M acetic acid was collected from $^{90}$Sr/$^{90}$Y in a concentration of 296 MBq/mL. The labelling of the particles with Y-90 was performed at pH 5.5 in acetate buffer with agitating for 60 min at room temperature. The labelled albumin suspensions were centrifuged at 6000 rpm for 15 min. Labelling yields was controlled using centrifugation, filtration and compared with paper chromatography, which is developed in the Tris Acetic EDTA. In this system, the unbound of Y-90 migrates to an Rf of 0.9-1.0 and the radiolabeled albumin particles remains at the point of origin (Rf = 0). The size of $^{90}$Y-albumin particles was compared with the albumin particles in the original solution to be sure that they didn’t change during the labelling treatment. The radiolabelling yields were more than 80% after centrifugation.
RESULTS OBTAINED

The major results obtained from our research can be summarized as follows:

1. The $^{90}$Sr/$^{90}$Y Generator: The stage #1 of the $^{90}$Sr/$^{90}$Y generator is needed to operate for 5 hours. Then the solution is transferred from the second chamber to the third chamber by using syringer 3 or the syringer 2. In the stage #1, the separation yield was determined and found to be around 80%. In the stage #2, transport of Y-90 was 95% in about 6 hours. Quality control of $^{90}$Y was carried out by the EPC technique as well as radioactive decay method followed by half life measurement. The radionuclidic purity of $^{90}$Y sample was more than 99.9999% with $^{90}$Sr contamination less than 0.001% which is far below the permissible level. Figs. 3, 4 and 5 shows the activity profile as a function time using the SLM generator develop in-house at 20 and 50 mCi levels. After second stage operation radiopharmaceutical grade of $^{90}$Y-acetate can be collected and used for radiolabelling biomolecules (Fig. 6).

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**Fig. 3.** Transport of $^{90}$Y from $^{90}$Sr- $^{90}$Y mixture at 20mCi level in stage -1
Fig. 4: Transport of $^{90}\text{Y}$ from $^{90}\text{Sr}$-$^{90}\text{Y}$ mixture at 50 mCi level in stage -1

Fig. 5: Transport of $^{90}\text{Y}$ from 4M HNO$_3$ medium to 1M acetic acid in Stage 2
Quality control of the Yttrium-90 was carried out for each lot of the $^{90}$Y product. The radiochemical purity was always found to be greater than 99.999% with $^{90}$Sr contamination less than 0.001% which is far below the permissible level (Fig. 7).

This radioisotope is suitable for radiolabelling biomolecules such as Rituximab, DOTA Octreotide, Albumin.

**STAGE-1 OPERATION**

In the first stage, $^{90}$Sr-$^{90}$Y mixture at equilibrium adjusted to pH 1-2 as described above was used as feed in compartment-1. PTFE support after impregnating with PC88A (60% in n-dodecane) was used for selective transport of $^{90}$Y. Nitric acid (4.0 M) was used as receiver phase. It took about 6 h to transport 93.61% $^{90}$Y activity in 4.0 M HNO$_3$. The results from the experiments are given in Table 1.
Table 1: Transport of beta activity due to $^{90}$Y in stage-1

Experimental conditions:
Feed (Compartment-1): $^{90}$Sr-$^{90}$Y mixture at equilibrium in nitric acid solution adjusted to pH 1-2
Membrane support: PTFE; 
Carrier: 60% PC88A in n-dodecane; 
Receiver (Compartment-2): 4.0 M HNO$_3$; 
Volume of each compartment: 5 mL.

<table>
<thead>
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<th>Time (h)</th>
<th>Beta activity in (mCi)</th>
<th>Percentage of beta activity due to $^{90}$Y transported in Compartment-2</th>
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</tr>
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<td>6</td>
<td>98.76</td>
<td>93.19</td>
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Separation yield in STAGE -1= 93.19%

Transfer of the feed solution in compartment-1 from the glass vial kept inside the lead chamber was carried out using a semi automated system consisting of silicon tubing attached with syringe at one end and rubber cork at the sampling ports. Similar mechanism was used for transferring the $^{90}$Y product in 4.0 M HNO$_3$ from first stage (compartment-2) to Compartment-3 to be used in the second stage. The system is shown in Fig. 8.

STAGE-2 OPERATION: 4.0 M nitric acid containing $^{90}$Y was removed from compartment-2 and introduced in compartment-3 of the second stage in which 0.8 M CMPO in n-dodecane based SLM was used to transport $^{90}$Y selectively to compartment-4 in which 1 M CH$_3$COOH was used as receiver phase. The results of $^{90}$Y transport in stage-2 are given in Table 2. The separation yield in the second stage is found to be 90.45% in 4 h.
Table 2: Transport of beta activity due to $^{90}$Y in stage-2

Experimental conditions:
Feed (Compartment-3): $^{90}$Y in 4.0 M nitric acid solution.
Membrane support: PTFE, Carrier: 0.80 M CMPO in n-dodecane.
Receiver (Compartment-4): 1.0 M CH$_3$COOH, Volume of each compartment: 5 mL.

<table>
<thead>
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<th>Time (h)</th>
<th>Beta activity due to $^{90}$Y in (mCi)</th>
<th>Percentage of beta activity due to $^{90}$Y transported in Compartment-4</th>
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Above results give the overall separation yield of above 84% for $^{90}$Y using the generator system.

2. Labelling Rituximab with Y-90

Coupling efficiency of cDTPA - to - Rituximab molar ratios of 1, 3, 5, 10, 20 at concentration of 5 mg/mL and 10 mg/mL Rituximab was around 82.0 - 53.5% and 78.2 - 24.4%, respectively (Fig. 9, 10). Coupling efficiency of ~63% at a 3:1 molar ratio results in an average of 2 groups per molecular. The conjugation mixture was diluted to about 0.2 mL with the bicarbonate buffer and loaded onto a PD-10 column (Sephadex G-25, Pharmacia, Biotech) (Fig. 10). After purification, the conjugation DTPA-Rituximab was collected and labeled with Y-90 in 0.5M acetate buffer, pH 5, at room temperature ($^{90}$Y obtained from $^{90}$Sr/$^{90}$Y generator developed in-house). The labeling yield was about 99%. The radiochemical purity of $^{90}$Y-DTPA-Rituximab was determined by ITLC and developed in 0.1M acetate at pH 6 as mobile phase. Radiochemical purity of $^{90}$Y-DTPA-Rituximab was more than 99%. The radiopharmaceutical has been tested for sterility, apyrogenicity and biodistribution.

![Fig. 9. Coupling efficiency of cDTPAa - to - Rituximab](image-url)
3. Labelling DOTATATE with Y-90

DOTATATE could be labelled with Y-90 with high radiochemical yields. After incubation and cooling at room temperature, the 20 μL of the labeled peptide was mixed with 200 μL of 0.25 mM DTPA for quality control on a SepPak C18 cartridge and compared with PC using 10% sodium acetate and methanol (30:70). pH optimization was performed at 10 μg of peptide, 37 MBq of Y-90 and the same conditions of temperature (90°C) and time (30 min). Results of optimum pH were ranged 4 to 5 (Fig. 11). Labelling yield was greater than 98% at 20 – 60 min incubation (Fig. 12). The radiochemical purity of the ⁹⁰Y- DOTATATE was ~99% (Fig. 13).
4. Labelling microaggregated albumin with Y-90

The diluted albumin particles in 0.5 M acetate buffer pH 6 could be labelled with $^{90}\text{Y}$-acetate using directed method. The radioactivities of Yttrium-90 were measured with a beta and gamma counters. Labelling yields of both of $^{90}\text{Y}$-Albumin and $^{90}\text{Y}$-MAASOL were greater than 80% (Fig. 14). The incubation time for the labelled reaction was ~60 min. (Fig. 15). We found that the suitable solvent for PC and ITLC of labeled microaggregated albumin and Yttrium-90 acetate is Tris Acetic EDTA. In this system, the free Y-90 moves to solvent front (Fig. 16). Radiochemical purity of the precipitation of $^{90}\text{Y}$-albumin was carried out by ITLC, developed in Tris Acetic EDTA buffer with more than 98% purity. MAASOL, Italy was also labeled with Y-90 at the same way and $^{90}\text{Y}$-MAASOL with high purity of 98% was collected (Fig. 17 and 18).
Fig. 14. Labelling yield of $^{90}$Y-Albumin (prepared in-house) and $^{90}$Y-MAASOL, Italy

Fig. 15. The incubation time of $^{90}$Y-albumin

Fig. 16. Radiochemical Purity of $^{90}$Y
   (ITLC developed in Tris Acetate EDTA solution)
CONCLUSIONS AND SUGGESTIONS

- Two stage SLM based $^{90}$Sr-$^{90}$Y generator for production of carrier-free $^{90}$Y could be successfully developed in house.

- Activities related to scale-up of $^{90}$Sr-$^{90}$Y generator using SLM technology was carried out at different levels (5, 20 and 50 mCi and 100 mCi).
- The EPC was applied to quickly and accurately determine the amount of $^{90}\text{Sr}$ in $^{90}\text{Y}$ in each lot of $^{90}\text{Y}$ separated.

- Rituximab was labelled with $^{90}\text{Y}$ collected from $^{90}\text{Sr}$-$^{90}\text{Y}$ generator developed in house. Rituximab was bound to the DTPA chelating agent using Hnatowich methods.

- DOTATATE and albumin particles were labelled with $^{90}\text{Y}$ collected from $^{90}\text{Sr}$-$^{90}\text{Y}$ generator developed in house. The labelling yields were high and radiochemical purity was greater than 98%. In order to determine radiochemical purity of $^{90}\text{Y}$- albumin, we found the new solution for analyses the bound and the free using the TAE system as mobile phase in PC and ITLC.

- In future, studies will mainly focused on the stabilities of the products, invivo and invitro stabilities of $^{90}\text{Y}$-biomolecules and their bio-distribution. In this regard expertise from Italy and Germany is needed.

- We expect IAEA’s support in supplying the equipment viz. Autoradiographic System, Cyclone Packard, PerkinElmer for preclinical evaluation in the year 2010.
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