ASTATINE-211 RADIOCHEMISTRY: THE DEVELOPMENT OF METHODOLOGIES FOR HIGH-ACTIVTY LEVEL RADIOSYNTHESIS

Final Progress Report
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A. OBJECTIVES AND SPECIFIC AIMS

Targeted radionuclide therapy is emerging as a viable approach for cancer treatment because of its potential for delivering curative doses of radiation to malignant cell populations while sparing normal tissues. Unfortunately, the current impact of targeted radiotherapy in the clinical domain has remains limited. In many cases, potentially useful molecular targets and labeled compounds have already been identified; however, putting these concepts into practice has been impeded by limitations in radiochemistry methodologies. A critical problem is that the synthesis of therapeutic radiopharmaceuticals provides additional challenges in comparison to diagnostic reagents because of the need to perform radio-synthesis at high levels of radioactivity. This is particularly important for a-particle emitters such as ^{211}At because they deposit large amounts of energy in a highly focal manner. ***The overall objective of this project was to develop convenient and reproducible radiochemical methodologies for the radiohalogenation of molecules with the α-particle emitter ^{211}At at the radioactivity levels needed for clinical studies.***

In this project, we attempted to address critical ramifications of this characteristic in the radiochemistry of targeted radiotherapeutics – the deleterious effect of high radiation fields on radiopharmaceutical synthesis and molecular integrity. Moreover, a well known but seldom reported characteristic of ^{211}At chemistry is that yields for electrophilic astatination reactions decline as the time interval after radionuclide isolation from the cyclotron target increases. This is a critical problem that must be addressed if cyclotrons are to be able to efficiently supply ^{211}At to remote users. Our studies confirmed that this phenomenon was related in large part to free-radical induced transformation of the ^{211}At to a lower oxidation state.

Because of the widespread interest in labeling antibodies (mAbs), antibody fragments and peptides with ^{211}At, our proposed work plan initially focused on two reagents that we have developed for this purpose: *N*-succinimidyl-3-[^{211}At]astatobenzoate (SAB) and *N*-succinimidyl 3-[^{211}At]astato-4-guanidinomethylbenzoate (SAGMB), both synthesized from their corresponding trialkylstannyl precursors. Investigations of the effects of radiolysis on the synthesis of SAB and SAGMB as a function of radiation dose and dose rate included evaluations of the effects of solvent, pH, oxidant and nature of the precursor on labeling yields at high activity concentrations. A limitation of SAB and SAGMB as well as other current procedures for labeling mAbs with ^{211}At is that less than 1 in 200 mAb molecules are labeled. Clearly, the impact of the low specific activity of ^{211}At-labeled mAbs can be significant, particularly for molecular targets where the number of receptors present per target cell is relatively low. As a model mAb for use in optimizing specific activity of ^{211}At labeling, we propose to utilize trastuzumab (Herceptin®). Our rationale for this choice was based in part on the fact that we have already developed a theoretical model and performed extensive *in vitro* experimentation demonstrating the dramatic effects of ^{211}At-labeled trastuzumab specific activity on its cytotoxicity.

Part of our strategy was the use of synthetic precursors immobilized on polymeric

resins or perfluorous and triarylphosphonium supports. Their use eliminated the need for a purification step to separate unreacted tin precursor from labeled product and hopefully provide a simple kit technology that could be utilized at other institutions. Furthermore, with ^{211}At, use of these supports might facilitate the development of a process for synthesizing ^{211}At-labeled radiopharmaceuticals *in situ* as the ^{211}At is being distilled from the cyclotron target. Because of the high levels of energy deposited in the reaction mixture, resulting in very high concentrations of free radicals, developing methodologies for ^{211}At was challenging. Nonetheless, our studies using a tin precursor anchored to a solid support for the synthesis of *meta*-[^{211}At]astatobenzylguanidine (MABG) gave higly encouraging results and suggested that this may be a promising approach for performing high activity level radioastatination reactions.

In order to achieve our goal of developing radiochemical methodologies that will help advance the field of targeted radionuclide therapy, the following specific aims were proposed:

Specific Aim 1: To optimze methods for ^{211}At production and isolation of ^{211}At from cyclotron targets. We shall focus on cryotrapping methodologies that are amenable to use as reactors for subsequent radiolabeling procedures.

Specific Aim 2: To develop convenient and reproducible methodologies for high activity level and high specific activity radiohalogenation of biomolecules with ^{211}At. Yields for the synthesis of the protein acylation agents SAB and SAGMB will be evaluated as a function of radiation dose and dose rate. If successful, we shall determine whether our most successful reaction conditions can be applied to the synthesis of MABG and other ^{211}At-labeled compounds.

Specific Aim 3: To develop a procedure for extending the shelf-life of ^{211}At beyond a few hours so that this radionuclide can be utilized at centers remote from its site of production. Our work to date suggests that with time, radiation-induced alterations in astatine oxidation state occur and that it might be possible to circumvent this through the addition of an appropriate stabilizer.

Specific Aim 4: To work out high activity level synthesis methods for utilizing support immobilized tin precursors for ^{211}At labeling, with MABG serving as a model compound. The nature of these studies will be similar to those performed in Specific Aim 2. If successful, we shall determine whether polymer supported synthesis can be utilized for other compounds of clinical interest such as SAB and SGMAB.

B. RESULTS

High-Level Production of Astatine-211 and its Use for Labeling mAbs at Activities Required for Targeted Radiotherapy Trials in Patients

Although a strong rationale has existed for some time for initiating clinical trials with ^{211}At-labeled compounds, patient studies had been impeded by the lack of appropriate methodologies for producing clinically relevant levels of ^{211}At-labeled radiopharmaceuticals. There are two aspects to this problem. First, cyclotron targetry and ^{211}At purification systems are needed to provide large quantities of ^{211}At (greater than 50 mCi) in appropriate chemical form for chemical manipulation. And second, the development of labeling and purification procedures that are appropriate for high-level syntheses under conditions where radiolytic decomposition may play a role are required. We have developed procedures that have been implemented for the production of clinical levels of ^{211}At-labeled mAbs. These methodologies have permitted the initiation of the first clinical trial of a ^{211}At-labeled endoradiotherapeutic agent, ^{211}At-labeled human/mouse chimeric anti-tenascin 81C6 mAb. A total of 16 batches of ^{211}At-labeled chimeric 81C6 have been prepared for clinical use, 12 of which were administered to glioma resection cavity patients. Three additional runs were successful; however, they were not administered because of clinical complications. The last run yielded a preparation with acceptable quality control characteristics; however, insufficient ^{211}At-labeled mAb was available for injection because of the retention of high activity levels in the reaction vessel.

Table 1. Production and distillation of ^{211}At for clinical studies

Run	Beam Current (mA)	Irradiation Time (min)	Target Activity (mCi)	Distilled Activity (mCi)	Distillation Yield (%)
1	52	90	60	36	72
2	52	90	53	34	79
3	51	120	76	41	69
4	55	120	82	49	71
5	55	120	89	39	61
6	53	120	78	40	74
7	52	120	93	39	67
8	57	180	127	75	66
9	53	180	123	74	75
10	50	240	143	98	88
11	60	240	144	74	71
12	50	180	121	58	74
13	55	240	178	91	68
14	55	270	174	101	73
15	55	240	172	83	64
16	55	240	143	61	68

Astatine-211 was produced at the Duke University Medical Center cyclotron by bombarding natural bismuth metal targets with 28.0-MeV a-particles using the ^{209}Bi(a, 2n)^{211}At reaction. The MIT-1 internal target system specifically designed for the production of ^{211}At was used. A detailed description of the target plate and modified distillation system, as well as associated methods, is presented in the Experimental Design and Methods Section. These ^{211}At production runs utilized beam currents of 50-60 mA a-particles and irradiation times of 1.5 to 4.5 hr (**Table 1**) with a mean yield of 0.75 ± 0.07 mCi/mA-hr. No significant differences were observed in ^{211}Atproduction as a function of beam current or irradiation time. The maximum level of ^{211}At that was produced was 178 mCi after a 4-hr irradiation at 55 mA. The decay-corrected distillation yield was 67 ± 16%.

Synthesis of *N*-succimimidyl 3-[^{211}At]astatobenzoate (SAB) was begun between 5 and 40 minutes after the end of the distillation, depending on whether the ^{211}At production was performed during the day, or for the longer duration runs, beginning at midnight. SAB was prepared by reacting in a glass vial the ^{211}At in chloroform, *N*-succinimidyl 3-(tri-*n*-butyl-stannyl)benzoate, *tert*-butylhydroperoxide, and glacial acetic acid. Instead of purifying SAB by HPLC, a modification of our previously described silica gel Sep-Pak™ cartridge method was used. The pH of a 10 mg aliquot of chimeric 81C6 was adjusted to a final pH of 8.8-9.2 by the addition of saturated borate buffer in a 1:1 volume ratio. The mAb was transferred to the vial containing SAB and incubated at room temperature for 15 min. The reaction was terminated by the addition of 0.2 M glycine in borate buffer. The ^{211}At-labeled chimeric 81C6 was purified by size-exclusion chromatography using a gas-sterilized, 1.5 × 10 cm borosilicate glass chromatography column loaded with Sephadex G-25.

Yields for the synthesis of SAB, which eluted in the 30% ethyl acetate in hexane fractions, averaged 54 ± 10%. The mean fraction of activity eluted in the hexane and 8% ethyl acetate in hexane fractions, presumably representing an At^{o} species and an astatinated p-complex with the tin precursor aromatic ring, was 7 ± 3% and 27 ± 8%, respectively. The mean activity retained on the column, most likely in the form of [^{211}At]astatide, was 12 ± 6%. The radiation dose deposited in the chloroform solution prior to initiation of the synthesis ranged from 12,500 to 394,300 cGy, and the dose delivered during the reaction ranged from 60,350 to 159,600 cGy. Because of the potential for radiolytic effects on the radioastatodestannylation reaction, the correlation between these radiation absorbed doses and SAB yield was investigated. There was no correlation between SAB yield and the radiation dose received by the reaction mixture. No correlations were found between the radiation dose delivered prior to and during the reaction and the fraction of activity either retained on the cartridge or eluted in the hexane, 8% ethyl acetate in hexane or 30% ethyl acetate in hexane fractions.

The radiolabeling yield for the coupling of SAB to chimeric 81C6 was 76 ± 8% after a 20 min reaction at room temperature. The radiation dose to the reaction medium ranged from 399 to 1196 Gy, and there was no significant correlation between radiation dose and coupling yield. The fraction of the ^{211}At activity in the clinical preparations that was

protein associated, determined by methanol precipitation, was 98.5 ± 1.0%. The fraction of ^{211}At activity which eluted with a retention time corresponding to intact IgG on size-exclusion HPLC was 96.0 ± 2.5%, with the remainder present as either aggregates (3.1 ± 2.5%) or low molecular weight impurities (0.9 ± 1.2%). All preparations had a pyrogen level <0.125 EU/mL and were determined to be sterile. After correcting for nonspecific binding, the average immunoreactive fraction for these 16 preparations was 83.3 ± 5.3%. When the immunoreactive fraction was plotted as a function of the radiation dose received by the mAb during radiolabeling and purification, regression analysis indicated that these data were best fit by the following equation: Immunoreactive fraction = 90.8% -0.010 × radiation dose (Gy). However, this slight decrease in immunoreactivity with increasing radiation dose was not a significant effect ($r^2 = 0.29$).

In summary**,** methods and procedures have been developed for the production of high levels of ^{211}At and for the preparation of sufficient ^{211}At-labeled chimeric 81C6 to permit clinical evaluation of this promising therapeutic radiopharmaceutical. We are working on further refinements of these methodologies to increase the available activity of ^{211}At-labeled mAb per mCi ^{211}At produced, and reduce total synthesis and purification time. Nonetheless, using our current methods, we have demonstrated for the first time that it is feasible to produce sufficient levels of ^{211}At-labeled compounds to perform endoradiotherapeutic investigations in patients.

Effects of ^{211}At Alpha-Particle Radiolysis on the Chemical Behavior of ^{211}At

The labeling methods described above provided reproducible results for 2-6.7 mCi doses of labeled mAb; however, with the exception of one run in which a 10 mCi dose was prepared, yields plummeted at higher activity levels. Two problems were encountered. First, the yield of SAB decreased to 30-40%. And second, after the reaction of SAB with the mAb, 40-60% of the ^{211}At was retained on the walls of the reaction vessel and the immunoreactivity of the labeled mAb was unacceptable. For these reasons, the clinical trial was stopped despite the promising responses that were obtained. These difficulties provided motivation for the studies described below, in which the effects of radiolysis on the chemical behavior of ^{211}At at high activity levels were investigated.

The fact that ^{211}At-labled compounds would by necessity generally be utilized at locations distant from a ^{211}At production site presents a major challenge to the chemist because under these circumstances, the astatine activity frequently arrives in a form that results in unacceptable labeling yields. The diminishing efficiency of electrophilic astatination reactions with the passage of time is well known, even when ^{211}At is utilized at its site of production. These difficulties likely relate to the cumulative effect of ^{211}At α-particles depositing large amounts of decay energy in a highly localized manner. The next set of experiments were directed at evaluating the effect of radiation dose on the astatine species present before initiation of a labeling reaction and the potential role of these molecules in the efficiency of SAB synthesis (Pozzi and Zalutsky, 2007). The distribution of astatine species present in methanol was determined by reversed-phase HPLC using radiation doses in the range of 500-12,000 Gy.

HPLC analysis indicated that essentially all of the ^{211}At activity eluting from the column was found in two peaks eluting at 6.2 and 10.7 min, designated hereafter as At(1) and At(2), respectively. The first peak, observed at 6.2 min, corresponded to that observed in freshly-prepared ^{211}At/methanol solutions and those exposed to doses of less than 1,500 Gy. The

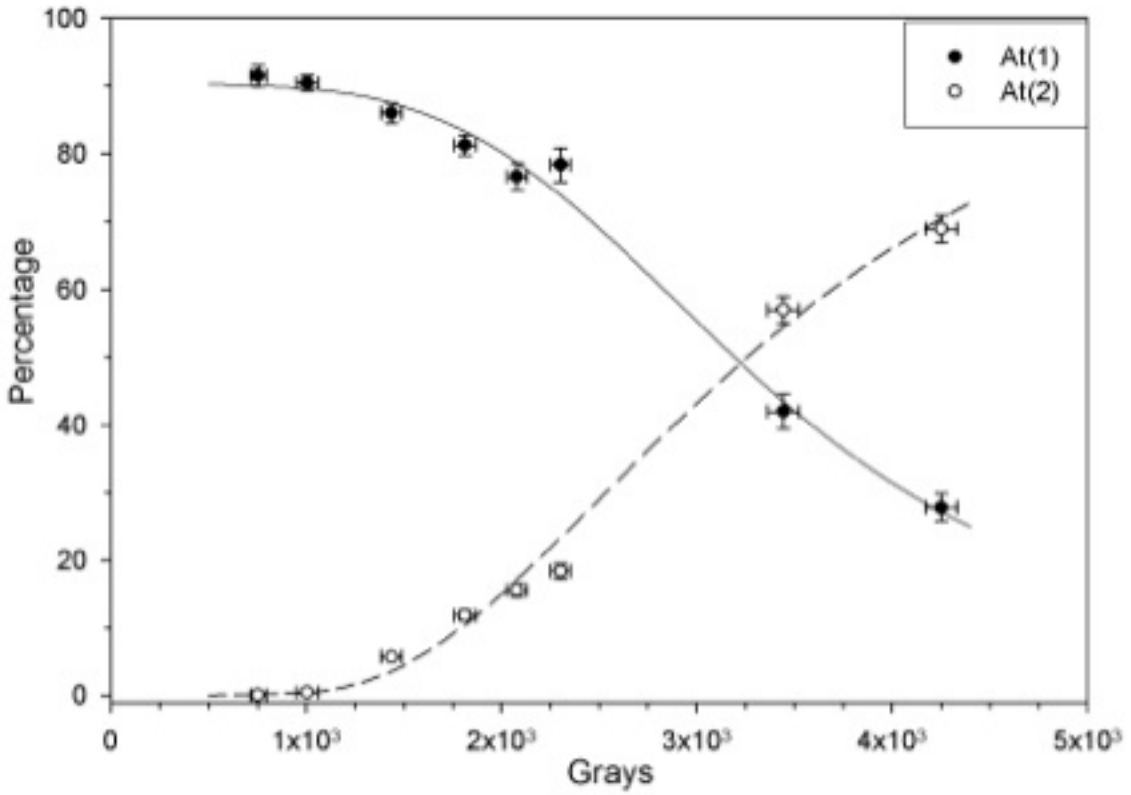

Figure 1. Relative amounts of At(1) and At(2) in MeOH (without HOAc) *vs.* radiation dose to solvent.

effect of radiation dose on ^{211}At/methanol solutions was further evaluated by taking aliquots at successive time periods, yielding increasing calculated radiation doses. The results for an experiment performed with an initial activity concentration of 43.5 MBq/540 µL are shown in **Figure 1**. The distribution of eluted ^{211}At activity between the two HPLC peaks clearly was dependent on the radiation dose received by the methanol; but not in a linear fashion. The contribution of At(2) increased gradually until about 2,200 Gy and then increased more rapidly, becoming the predominant species at radiation doses above about 3,200 Gy. Because acetic acid is a standard component in electrophilic astatination reactions, the ^{211}At species generated in ^{211}At/methanol solutions containing acetic acid (0.67 mol/L) was evaluated. At all doses investigated (1,000 to 20,000 Gy), at least 95% of eluted ^{211}At activity was found in the At(2) form. Thus, at low radiation doses, astatine is present in different forms in methanol with or without acetic acid while at higher radiation doses, At(2) predominates in both cases.

Based on the fact that methanol radiolysis in known to generate reducing species, particularly at acidic pH, these observations suggested that At(2) is a reduced form of astatine, most likely astatide. To investigate this possibility further, ^{211}At/methanol was treated with sodium sulfite. Treatment with this reducing agent decreased the fraction of eluted activity in the At(1) peak from 46% of eluted activity to less than 1%, whereas the contribution from the At(2) peak increased from 52% to 90%. Addition of the reducing agent resulted in almost complete conversion of At(1) to At(2) in 5 other paired experiments performed at different initial activity concentrations, providing further evidence that At(2) represents a reduced form of astatine. Furthermore, analysis of Na[^{131}I]iodide/methanol solutions under the same HPLC conditions showed that iodide eluted as a single peak at 10.2 min, a retention time quite similar to that observed for At(2). To better understand the chemical nature of the two species observed in the HPLC profiles, we analyzed ^{211}At/methanol solutions by electrophoresis. **Figure 2** is a direct comparison of the HPLC and electrophoresis profiles obtained from the same methanol solution after exposure to ^{211}At. Two peaks with similar relative magnitudes are observed with both techniques. In the electrophoresis profile, the larger peak is found at the origin, indicating that it is neutral, whereas the smaller peak migrates toward the anode, indicating that it is negatively charged. The electrophoretic behavior of an aliquot of the At(2) activity isolated by HPLC also was evaluated. A single species migrating toward the anode was observed, confirming that At(2) is a negatively charged species.

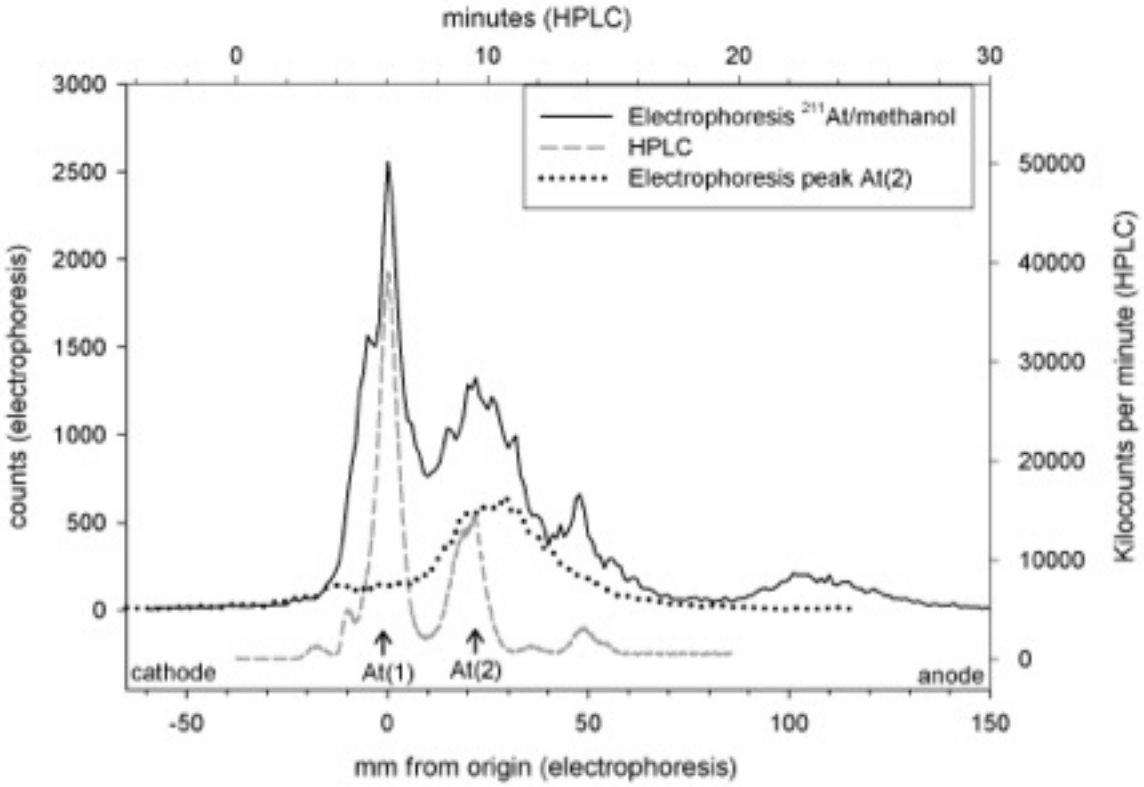

Figure 2. Analysis of ^{211}At/MeOH solution by HPLC (dashed line) and electrophoresis (solid line). Isolated At(2) peak also analyzed by electrophoresis (dotted line).

The peak corresponding to At(1) predominates in fresh ^{211}At/methanol solutions at low radiation doses, conditions where electrophilic astatination reactions give high reaction yields. Indeed, SAB yields and the fraction of ^{211}At present in methanol as At(1) exhibited similar dose dependent behavior. The emergence of a reduced form of astatine, At(2), at higher radiation doses could account for the decline in SAB yields under these conditions. These results demonstrate for what is to our knowledge, the first time, another significant impediment to successful astatine chemistry: alteration in the chemical form of the ^{211}At with increasing radiation dose from forms suitable for electrophilic labeling reactions to forms that are not. In methanol, the optimal solvent identified to date for therapy-level astatodestannylation, astatine is present at low radiation doses in a form from which At+ can readily be generated, perhaps stabilized in a complex with methanol. With increasing radiation dose deposition to the solvent, conversion to a reduced species, probably astatide, occurs, accounting for the decline in labeling yields at elevated radiation doses.

This also suggests a potential compensatory strategy: treatment of At(2) with an

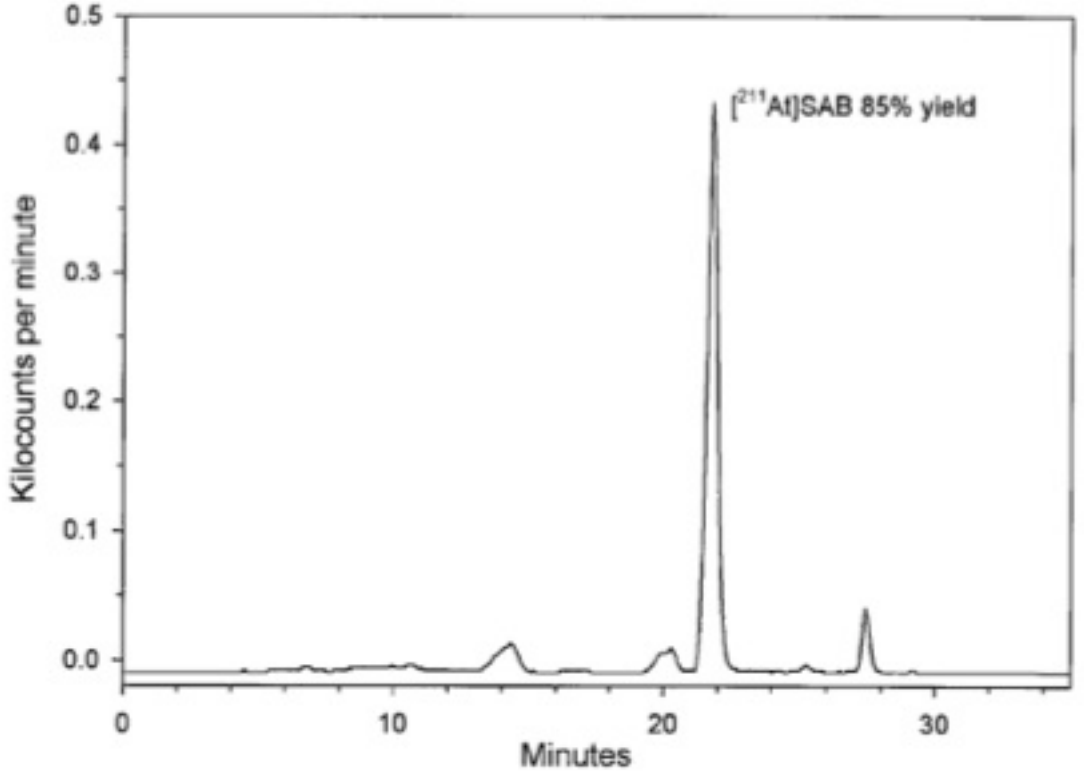

Figure 3. HPLC Chromatogram of the electrophilic reaction product using ^{211}At in methanol that was stabilized with 100 µg NCS, stored for 23 hr and reacted with BuSTB to form SAB. Calculated radiation dose received during storage and reaction was 50,046 Gy.

oxidant such as NCS to generate a reactive electrophilic astatine species. Although adding NCS to the reaction mixture resulted in conversion of a greater fraction of At(2) to SAB, the process was not efficient. For this reason, we developed an alternative strategy that involved "stabilization" of the astatine through addition of an oxidant earlier in the radiochemistry process. In this approach, the critical step is to add the oxidant immediately to the ^{211}At as it is distilled from the cyclotron target. Generally, this was done by adding NCS to the methanol solution that is utilized to recover the ^{211}At from the PEEEK tubing trap. A variety of NCS concentrations were investigated and 100-200 μg was found to be the optimal level. As an example, ^{211}At was distilled and trapped in a methanol solution containing 100 μg of NCS. The solution was stored at room temperature for 23 hr such that the radiation dose delivered to the solution by ^{211}At was in excess of 50,000 Gy. The tin precursor for SAB synthesis, BuSTB, was then added and the reaction allowed to proceed. As shown in **Figure 3**, even at this exceptionally high dose level, SAB was synthesized in 85% yield. To put this in perspective, without astatine stabilization, SAB synthesis yields typically were 15% or less even at radiation doses of only 3,000 Gy.

We believe that the ^{211}At stabilization strategy described above could have a major impact on the field because it overcomes many of the current obstacles to the development of ^{211}At-labeled targeted therapeutics from the radiochemistry perspective. The detrimental effects of radiolysis on the labeling chemistry are overcome, even at α-particle radiation doses previously considered prohibitive. As a result of stabilization according to the present invention, a substantially increased amount of α-particle emitter radioactivity can be incorporated into the desired α-particle emitter labeled compounds, such as those formed by the reaction of the α-particle emitter with an organometallic precursor. The correspondingly high levels of radioactivity of the α-particle emitter labeled pharmaceuticals made from these precursors are suitable for practical therapeutic applications. Because of the stabilizing effects according to the present invention, α-particle emitter labeled pharmaceuticals can be prepared well after the time (or far away from the site) of production of the α-particle emitter. A significant practical advantage of the present invention therefore resides in the ability to store the α-particle emitter in solution at high radioactivity levels and/or for extended periods, without suffering an associated loss in the radiochemical reaction yield of the α-particle emitter labeled compound.

Based on experiments performed to date and given the doses that can now be stored in astatine-containing solutions, with the astatine being made at Duke University's cyclotron, astatine can be sent to remote locations, and even overseas. This advance is particularly important because there are only a few cyclotrons (less than 5 in the United States) capable of producing therapeutic levels of ^{211}At. In the experiment where astatine was stored in a solution receiving up to 50,046 Gy, this would be equivalent to sending 35 mCi radioactivity in 500 μL for 5 hours, where 22 mCi would arrive on site. New York City could be reached from Duke University in this time. This 22 mCi of activity that a lab located at 5 hours from the Duke cyclotron could receive is almost the same as that which would be needed to start the chemical labeling procedure for preparing an ^{211}At-labeled mAb at a radioactivity level would be sufficient for administration to patients.

C. PUBLICATIONS

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