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Quality control of actinium-225 and ^{225}Ac-radiopharmaceuticals: Francium-221 to be or not to be?

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Abstract

Background Actinium-225 radiopharmaceuticals have drawn great interest in cancer therapy due to their tumor-specific delivery of cytotoxic alpha-particles. Quality control (QC) is critical for these potent agents and there is currently no consensus for best practice of actinium-225 QC. Detection of actinium-225 ($T_{1/2}$ = 9.92 days) is challenging; however, the francium-221 ($T_{1/2}$ = 4.8 min, 218 keV) and bismuth-213 ($T_{1/2}$ = 45 min, 440 keV) gamma-emitting progenies facilitate the quantification. Multiple analytical methods were investigated for limit of quantification (LOQ), radiochemical yield (RCY%), and radiochemical purity (RCP%). Those were evaluated at both francium-221 and bismuth-213 secular equilibria, and compared for accuracy and precision. While each secular equilibrium has been amply explored for actinium-225 quantification, a direct comparison between the two approaches has sparsely been examined. The RCY% was evaluated using two TLC plate readers: a gas-filled proportional counter and a plastic silicon photomultiplier detector. TLC strips were also analyzed using Liquid Scintillation Counting (LSC), High Purity Germanium (HPGe), and NaI(Tl) gamma well counting. In absence of detectable radio-impurity, HPLC RCP% were correlated to RCY%. Finally, free actinium-225 spiking recoveries were evaluated in ^{225}Ac-radiopharmaceuticals.

Results The plastic silicon scanner resulted in RCY% differences between 30 min and > 5 h evaluations, whereas the gas-filled proportional counter, when varying voltages, showed minor differences. Similarly, HPGe-TLC demonstrated equivalent RCY% between both equilibria. The NaI(Tl), LSC- TLC, and HPLC-gamma counting significantly underestimated RCY% or RCP% at 30 min. Considering gamma well counting LOQ and ^{225}Ac-radiopharmaceutical low radioactive concentration, as low as 1% of free actinium-225 may be accurately detected in HPLC fractions. For TLC, LOQs were reported lower than measured in solution, free actinium-225 radioimpurity may be precisely measured as low as 0.5% of total content, except for the silicon detector.

Conclusion Five instruments have been tested for their linearity, sensitivity, accuracy and specificity to actinium-225 quantification using francium-221 and bismuth-213 equilibria. Francium-221 equilibrium was deemed acceptable for TLC RCY% using HPGe and gas-filled proportional counter. Gamma well counting, LSC, and plastic

silicon detector required bismuth-213 equilibrium for accurate RCY%. Low content free actinium-225 impurity was accurately reported for all methods except for the silicon detector. Overall, this investigation sheds light on the appropriate analytical methods for ^{225}Ac-radiopharmaceutical QC considering secular equilibrium.

Keywords Actinium-225 quality control, Liquid scintillation, High purity germanium, Gamma well counting, Thin layer chromatography, HPLC

Introduction

Actinium-225 radiopharmaceuticals have drawn a great interest in cancer therapy due to their tumor-specific delivery of cytotoxic alpha-particles. To date, > 20 clinical trials have been launched world-wide using ^{225}Ac-radiopharmaceuticals, including > 10 clinical trials actively recruiting in phase 2–3 (https://clinicaltrials.gov/). While ^{225}Ac-radiop harmaceutical development has exponentially grown over the last 10 years, several complications have hindered the progression to clinic. Particularly, the global actinium-225 supply is currently insufficient; however, manufacturing initiatives are in progress to support the needs of research and clinical trials. Precluded access to actinium-225 has led to expedited studies and little understanding of its specific detection. Consequently, there is currently no consensus for best practice in quality control (QC) of actinium-225 and ^{225}Ac-radiopharmaceuticals. This is a critical issue hampering the development of these potent agents.

The vast majority of nuclear medicine drug products (DPs) are diagnostic radiopharmaceuticals. Unlike PET or SPECT radionuclides, actinium-225 ($T_{1/2}$ = 9.92 days) gamma-ray energies are too weak to be accurately detected with commonly used radiopharmacy equipment. Alternatively, the gamma-emitting progenies francium-221 ($T_{1/2}$ = 4.8 min, 218 keV, *%I*: 11.44) and bismuth-213 ($T_{1/2}$ = 45 min, 440 keV, *%I*: 25.9) have been adopted as the radionuclides of choice to quantify actinium-225 at their respective secular equilibrium (Fig. 1).

Following purification or separation of actinium-225 or labelled substance, secular equilibrium of the progenies—francium-221 or bismuth-213—is disturbed and must be reestablished to accurately quantify actinium-225. To determine the actinium-225 activity, quantification by francium-221 may be undertaken as early as 30 min post-separation and as early as 5 h for bismuth-213 (Fig. 1). Previous studies reported actinium-225 quantification based on the francium-221 gamma ray (218 keV) at 30 min post-separation (Hooijman et al. 2021, 2025, 2024; International Atomic Energy Agency 2024). This strategy was applied to radiochemical purity (RCP%) evaluation of actinium-225 DPs using High Performance Liquid Chromatography (HPLC) fractions which were gamma counted at 30 min following chromatographic separation (Hooijman et al. 2024). Using this strategy, the analytical method development of [^{225}Ac]Ac-PSMA-I&T, indicated significant differences (7–9%) between RCY% and RCP% when comparing the results from Thin Layer Chromatography (TLC) scanner to those of HPLC or TLC counted using HPGe (Hooijman et al. 2021). This inter-instrument discrepancy poses challenges in accuracy. A difference of > 5% in RCP% is problematic for the DP release because RCP% values ≥ 95% are required for release (International Atomic Energy Agency 2024).

In contrast, actinium-225 radiopharmaceutical QC exclusively based on bismuth-213 secular equilibrium quantification using both TLC and HPLC analyses were reported within a < 5% difference (Abou et al. 2022). While this is an improvement to the

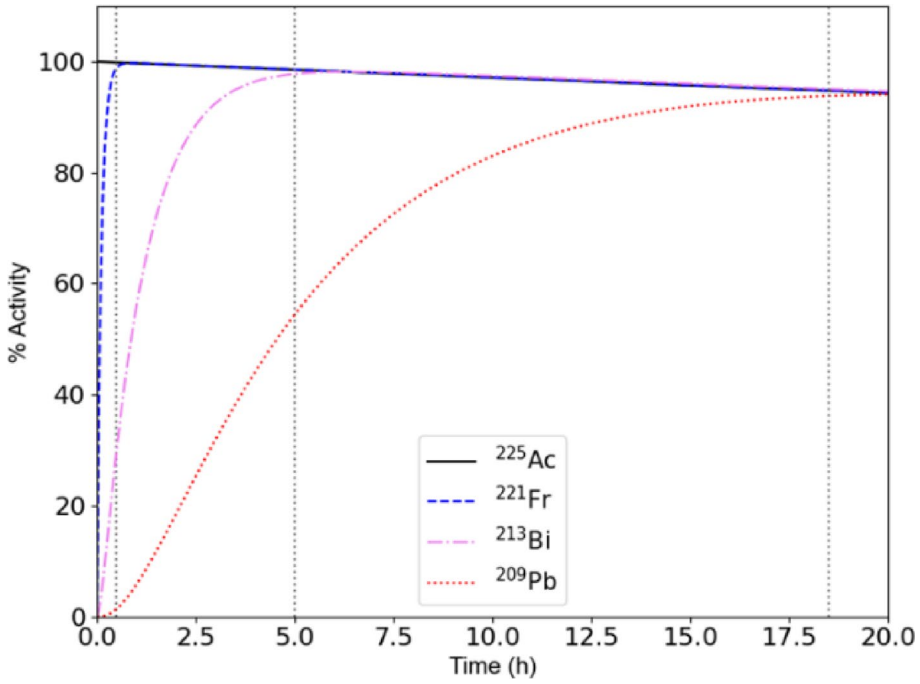

Fig. 1 Actinium-225 decay and secular equilibrium of francium-221 reached at 30 min (98%) and bismuth-213 at 5 h (> 98%)

francium-221 only detection method, a difference of 2–5% may still not satisfy accuracy and precision QC criteria for ^{225}Ac-radiopharmaceuticals as recommended (Gillings et al. 2020; ICH guideline Q2(R2)).

In the present investigation, multiple analytical methods are compared for actinium-225 quantification using francium-221 (at 30 min) and bismuth-213 (> 5 h) detection. Linearity and limit of quantifications (LOQs) of actinium-225 in solution and spotted on TLC strips were defined utilizing: liquid scintillation counting (LSC), high purity germanium (HPGe), and NaI(Tl) gamma well counting. TLC evaluations were conducted using TLC scanners equipped with a gas-filled proportional counter and a plastic silicon photomultiplier detector, as well as a "cut and count" strategy using LSC, HPGe, or NaI(Tl) gamma well counting.

Once the instrument capabilities were defined with respect to the actinium-225 detection limit, DPs were studied to assess their radiochemical yield (RCY%) and radiochemical purity (RCP%). Determinations based on francium-221 equilibrium were compared to those of bismuth-213 and evaluated for accuracy and precision. Additional attributes were examined, focusing on the specificity and sensitivity of free actinium-225 quantification in the DP. Recommendations for radiopharmaceutical QC indicate a detection down to 0.5% impurities (Gillings et al. 2020). In previous investigations of ^{225}Ac-radiopharmaceuticals, reports suggested a detection limit as low as 1% for free actinium-225 impurities (Hooijman et al. 2025). The low radioactive concentration of an ^{225}Ac-radiopharmaceutical requires achieving an adequately low LOQ. In this report, free actinium-225 spiking assays were conducted, and accuracy was evaluated for each instrument.

The evaluation of RCY% and RCP% at francium-221 and bismuth-213 equilibrium is meant to shed light on analytical methods accuracy and precision of actinium-225

quantification. While existing methods have been described using francium-221 or bismuth-213 equilibrium, a direct comparison of each evaluation has sparsely been examined. Reports of equivalence between the two equilibria evaluations remains to be understood in order to verify the most appropriate one for ^{225}Ac-radiopharmaceutical characterization. Similarly, specificity and sensitivity of actinium-225 detection merits investigations in order to clarify which instrument is best suited in consideration of the low ^{225}Ac-radiopharmaceutical radioactive concentration. Altogether, the examination of each instrument quantification limits, the RCY% and RCP% based on francium-221 (at 30 min) versus bismuth-213 (> 5 h) detection and the quantification of low-level free actinium-225 impurity are meant to better support the understanding of ^{225}Ac-radiopharmaceutical QC.

Material and methods

All handling of radioactive substances was performed by trained personnel following ALARA principles using adequate equipment and protection at NorthStar Medical Radioisotopes under an approved license issued by the Wisconsin Department of Health Services.

All chemicals utilized in this study were purchased at Fisher scientific (USA). Actinium nitrate, supplied by U.S. Department of Energy Isotope Program, was transferred into chloride with a confirmed radionuclidic purity > 99.8% per the certificate of analysis. The list of radiolabeled constructs utilized for each experiment are tabulated in Suppl. Info. Table 1A, B. None of the drug preparation described in this manuscript were utilized for patient administration.

Linearity and limit of quantification (LOQ)

Actinium chloride ([^{225}Ac]$AcCl_3$) was dissolved in 0.1 N HCl or DTPA 10 mM (pH 5–6) and split for the preparation of standard solutions. High purity germanium and dose calibrator measurements were undertaken to evaluate the exact actinium-225 activity amounts of dissolved source before each dilution. All instruments were calibrated and verified for suitability performance utilizing radionuclide standards provided by the manufacturer or purchased at Eckert & Ziegler (E&Z). The LSC (Hidex 300 SL, LabLogic) was calibrated using an unquenched standard set (SET-H3C14-LSC-20FST, E&Z). The HPGe (GEM10P-70, Ortec®, GFG_ICS-P4 cooler, Gamma vision) was calibrated for energy and efficiency using a mixed-radionuclide standard within a 20 mL vial geometry (7500 model E&Z). The NaI(Tl) gamma well counter (Hidex AMG) was calibrated daily using a cesium-137 source (Spectrum Techniques). The silicon detector TLC scanner (Scan-RAM 1™, LabLogic) was calibrated using a 3-source cesium-137 calibration plate provided by the manufacturer (LabLogic). The gas proportional counter TLC scanner (AR-2000 E&Z) was calibrated using a carbon-14 plate (AR-4101A, E&Z).

Solutions of actinium-225 were prepared via gravimetric assessments for exact volume transfer. A serial dilution of actinium-225 solution was completed with varying radioactive concentration depending on the evaluated technique. Linearity and LOQ were measured in solutions and spotted on TLCs using LSC, HPGe, Gamma-well counting, and TLC scanners.

The LSC measurements were completed utilizing a topographical spectra algorithm discriminating beta from alpha emissions based on their pulse length index (PLI). Alpha

particles present longer pulse lengths to beta particles enabling some degree of radionuclidic differentiation. The alpha-emitting radionuclides discrimination is however limited by the low energy resolution of the instrument. In this case, linearity and LOQs were evaluated using gross alpha counting acquired as alpha count rate set at PLI 39. Serial dilution of actinium-225 solutions were prepared from 2.96 mBq/µL to 740 Bq/µL. A 5 µL aliquot of each solution was added to the liquid scintillant cocktail resulting in a clear limpid solution (10 mL, Ultima Gold AB™). Similarly, a 5 µL aliquot of standards (37 mBq/µL and 1.48 Bq/µL) was spotted on TLC strips. Strips were transferred into a vial containing 10 mL of liquid scintillant cocktail. Samples were mixed and stored in the dark for at least 15 min before acquisition for 1 min per sample.

For HPGe measurements, a 10 µL aliquot of actinium-225 solutions (1.85 Bq/µL to 498 Bq/µL) was diluted to 20 mL with water, repeating the calibration geometry. TLC strips were prepared as described above. Once spotted, the unmigrated sealed strip (5.5 × 1.5 cm) was secured in a 3D printed holder for standardized geometry on the detector. All samples were measured directly on the detector for 5 min with a dead-time never exceeding 10%. Net peak area counts were retrieved at 218 keV and 440 keV photopeaks and plotted for linearity.

Gamma well counting was acquired 1 min per sample utilizing three energy windows: 195–240 keV for francium-221 detection (218 keV), 350–530 keV for bismuth-213 (440 keV), and 15–530 keV for complete energy range. The solutions of actinium-225 (0.925 kBq – 37 kBq, 750 µL) were measured in a HPLC glass vial of 1.5 mL. Another linearity was completed using 20 µL solutions in a 1.5 mL polypropylene microcentrifuge tube. TLC linearity was completed spotting a 5 µL aliquot of actinium-225 solutions on TLC strips. All measurements were background corrected.

Linearity was evaluated as well utilizing two TLC scanners equipped with either a gas-filled proportional counter or a silicon photomultiplier tube. Actinium-225 solutions (10 µL) were spotted on strips, 37 Bq to 148 kBq for the silicon detector with 30 min acquisitions, and 7.4 Bq to 29.6 kBq activity was spotted for the gas-filled proportional counter measured 1 min at 1000 V. Operated at 1000 V high voltage, the gas-filled proportional counter measured gross alpha counting, or alpha-specific detection. The 1500 V voltage was set for mixed emission beta and gamma detection (Friedlander et al. 1981; Chase and Rabinowitz).

The linear regression was plotted for each instrument, verifying $R^2 > 0.99$ (Suppl. Figs. 1, 2). LOQ was defined following Eq. 1, per ICH Q2(R2).

$$\mathrm{LOQ} = 10 \times \mathrm{SDy\text{-}i/Slope\,(with\ SDy\text{-}i\ the\ standard\ deviation\ of\ the\ y\text{-}intercept)} \quad (1)$$

The later was calculated utilizing the lowest 5 activity amounts/counts of the linear regression using the LINEST function.

Drug product RCY% and RCP% evaluations using TLC and HPLC

Five instruments were utilized to measure the RCY% or RCP% of ^{225}Ac-labeled DPs using TLC or HPLC. Investigations using TLC scanners were undertaken comparing the performance of a plastic silicon detector to that of a gas-filled proportional counter. While the plastic silicon detector is mostly designed to detect beta- and gamma-emitting radionuclides, the gas-filled proportional counter enables a high voltage modulation

for alpha specific detection at 1000 V and a beta/gamma specific detection at 1500 V (Friedlander et al. 1981; Chase and Rabinowitz).

Other detectors such as LSC, HPGe, and Gamma well counting were considered for TLC analysis using a cut and count approach. Gamma well counting was utilized for both HPLC fractions and TLC measurements.

A DOTA-conjugated precursor, peptide or monoclonal antibody (Suppl. Info Table 1A, B), was radiolabeled using actinium-225 suspended in HCl (0.1 N). To eliminate possible [^{213}Bi]Bi-radiolabeled product interferences, the drug samples were evaluated > 5 h post-synthesis. The RCY% and RCP% were measured using TLC and HPLC, respectively. TLC may only quantify free isotopes in the DP, or RCY%. HPLC warrants radiolytic degradants identification and quantification, defining RCP%.

The TLC strips (11 × 1.5 cm) were migrated using citric acid (50 mM, pH 5, peptide construct) or EDTA (10 mM, pH 5, antibody construct). The developed strips were measured at 30 min or > 5 h post-development using TLC scanners. The acquisitions were performed using the gas-filled proportional counter 1 min per strip using either 1000 V or 1500 V. For the silicon detector, 20–30 min acquisitions were completed to obtain sufficient counts, and background corrected. The RCY% was calculated integrating selected regions of interest the unmigrated radiolabeled precursor (region 1, 0 < Rf < 0.5) and the free radionuclide impurity (region 2, 0.5 < Rf < 1), per Eq. 2.

$$\mathrm{RCY\%} = \mathrm{ROI}\ region\ 1 \times 100 / \left[\sum \mathrm{ROI}\ region\ 1\&2\right] \tag{2}$$

For LSC, HPGe, and NaI(Tl) gamma well counting TLC strips were cut in half after development at 55 mm from the edge. Prior to cutting, the radiolabeled precursor was verified retained at the spotted area post-development (Suppl. Figs. 3, 4). The bottom ½ strips ($0 < R_f < 0.5$) or the spotted section, was identified as the radiolabeled precursor; the top ½ strips ($0.5 < R_f < 1$) was identified as the free radionuclide impurities. Each ½ strips (top and bottom) were measured at 30 min and > 5 h post-TLC development using LSC, HPGe and gamma counting. LSC was conducted with 1 min acquisition following the same parameters previously described. HPGe was completed in 5 min acquisition with the ½ strips immobilized in a 3D-printed scaffold for fixed geometry on the detector. Activity quantification was completed using 218 keV francium-221 peak at 30 min post-development and 440 keV bismuth-213 peak at > 5 h post-development. Gamma well counting, 1 min acquisition, was completed at 195–240 keV (at 30 min); 350–500 keV (> 5 h) and 15–530 keV (> 5 h). RCYs% measured at 30 min were compared to that of > 5 h for each technique.

The HPLC/gamma well counting RCP% assessment was completed injecting 50 µL of DP in the HPLC (Thermo Scientific Vanquish™ Flex HPLC System) equipped with a Multiple Wavelength Detector and a fraction collector. The HPLC–UV and gamma counting of collected fractions were acquired for a [^{225}Ac]Ac-DOTA-peptide (identified #3F1, 3F2, 3F3). Samples were analyzed using a Xterra Phenyl column, 125 Å, 3.5 µm, 3.9 mm × 150 mm (Waters), with a controlled column temperature of 25 °C, and the autosampler at 4 °C. The UV-chromatograms were acquired at 225 nm, with a flow rate 0.4 mL/min. Mobile phase (MP) A: water and MP B: acetonitrile were both acidified using 0.1% v/v formic acid. A gradient was applied as followed: 0 and 12 min, MPA 77% to 65% and MPB 23–35%; 12 and 13 min, MPA 65% to 0% and MPB 35% to 100%; 13 and

17 min, MPA 0% and MPB 100%; 17 and 18 min, MPA 0% to 77% and MPB 100% to 23%; and 18 to 24 min, MPA 77% and MPB at 23%. Fractions were collected every 30 s over 24 min acquisition. Radiochromatograms were plotted from gamma counted fraction measured 1 min per fraction. Fractions were counted as early as 30 min after collection using francium-221 window; and 5 h after collection using bismuth-213 window. Count per minute (cpm) were background corrected. The RCP% was calculated following Eq. 2, integrating the sum of free actinium-225 and progenies cpm (peak 1–4 min) with that of the radiolabeled drug (Rt = 10–13 min). Radiolytic products were not detected however due to the low precursor content, detectability might be challenging (Fig. 2 and Suppl. Figs. 3, 4).

The RCY% and RCP% correlation study was completed using the silicon TLC scanner acquired > 40 min and the HPLC-gamma counted fractions measured both at bismuth-213 secular equilibrium.

Accuracy testing and free actinium-225 impurity spiking assay

The free actinium-225 impurity spiking assay was completed, as described by Gillings et al. (Gillings et al. 2020). Briefly, the DP was measured unaltered for RCP% and RCY%. Subsequently, the DP was split and spiked with various amount of free actinium-225 impurity, expressed as impurity % to total activity content.

Expected free actinium-225 quantification was calculated based on the initial impurity % in the unaltered DP batch combined with the % of added free actinium-225. The added free actinium-225 was precisely measured using gravimetry and HPGe. Expected free actinium-225% ranged from 3–14% after addition. A metric of instrument accuracy includes calculating the recovery in measured RCP% or RCY% with respect to expected value, as stated in Eq. 3 (Gillings et al. 2020). Recovery was found accurate when ranging between 90 and 110%.

$$\text{Recovery\%} = [\text{Measured RCP\% or RCY\%}] \times 100 / [\text{Expected RCP\% or RCY\%}] \quad (3)$$

Results

Linearity and limit of quantifications (LOQs)

Each instrument performance was evaluated for linearity and LOQs of actinium-225 in solution or spotted on TLC strips. Actinium-225 verified-activity solutions were measured according to instrument geometry calibration (LSC 10 mL, HPGe 20 ml). Gamma well counting was conducted under two different geometries 750 µL and 20 µL volume. All demonstrated linearity with $R^2 > 0.99$ (Suppl. Fig. 1). The LOQs were defined as 0.7 Bq for LSC, 15–56 Bq for HPGe, and 565–772 Bq for gamma well counting at 750 µL, and 11–92.5 Bq at 20 µL depending on the energy window (Table 1, Suppl Fig. 1).

A similar evaluation was conducted with activity spotted on non-developed TLC strips (0.37 kBq to 55.5 kBq). The LOQs were measured as low as 0.3 Bq for LSC, 15–36 Bq for HPGe, and 62–67 Bq for gamma well counting (Table 2, Suppl Fig. 2). The gas-filled proportional counter TLC scanner outperformed the silicon detector with a LOQ of 11.1 Bq at 1000 V.

Overall, these results place LSC, HPGe, and the gas-filled proportional counter TLC scanner as the most sensitive tools to detect < 37 Bq of free actinium-225 in DP whether

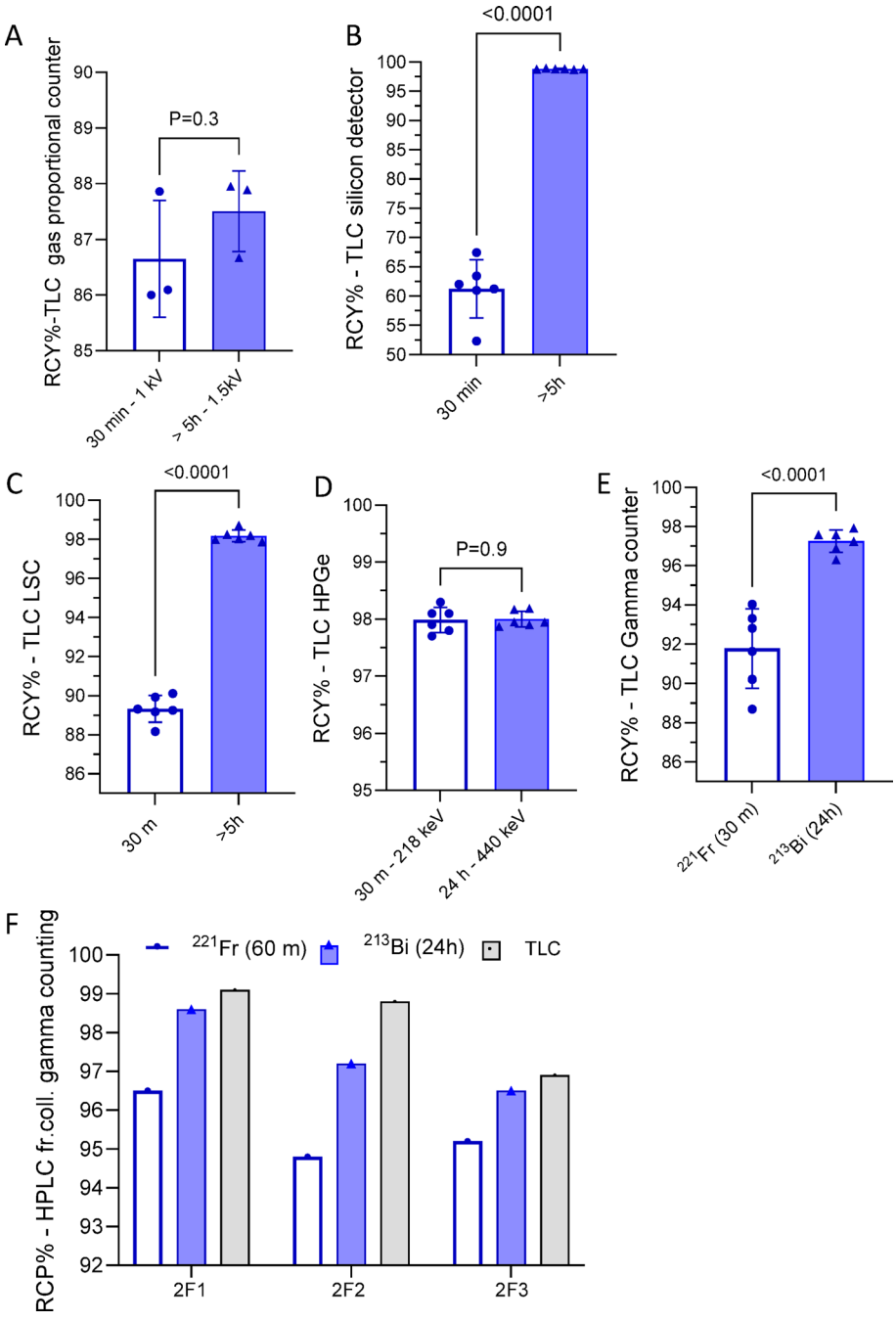

Fig. 2 RCY% determined using TLC either detected at francium-221 (30–60 min) or bismuth-213 equilibrium (> 5 h) measured with a TLC scanner equipped with **A** a gas-filled proportional counter; **B** a silicon detector; or a TLC cut and counted with **C** LSC; **D** HPGe; **E** gamma well counting. **F** RCP% of 3 different samples (2F1, 2F2, and 2F3) determined using gamma counting of HPLC-fraction collection at 60 min (218 keV) and > 5 h (440 keV), reported with TLC-RCY% at bismuth-213 equilibrium. Sample numbering and identifiers are listed in Suppl. Table 1B

in solution or spotted on TLC. The LOQs for actinium-225 in solution resulted in higher values than those spotted on TLC strips, suggesting a possible geometry effect.

Francium-221 versus bismuth-213 secular equilibrium

The suitability of techniques to determine accurate RCY% or RCP% was evaluated at francium-221 secular equilibrium, 30 min, versus bismuth-213 at > 5 h post-TLC

Table 1 LOQs of actinium-225 in solution, measured using LSC, HPGe, and gamma well counting. Gamma well counting was acquired with volumes of 750 µL and 20 µL under francium-221 (195–240 keV), bismuth-213 (350–530 keV), and open energy window (15–530 keV)

Solution	LSC	HPGe (keV)		Gamma well counter (keV) 750 µL			Gamma well counter (keV) 20 µL		
Detection	Alpha	218	440	195–240	350–530	15–530	195–240	350–530	15–530
LOQ (Bq)	0.7	15	56	772	642	565	92.5	40.7	11.1

Table 2 LOQ for actinium-225 spotted on TLC strips measured using LSC, HPGe, gamma well counting, and TLC scanners plastic silicon or gas-filled proportional detectors

TLC	LSC	HPGe (keV)		Gamma well counter (keV)			TLC scanner	
Detection	Alpha	218	440	195–240	350–530	15–530	Silicon	Proportional
LOQ (Bq)	0.3	36	15	62	67	64.7	1251	11.1

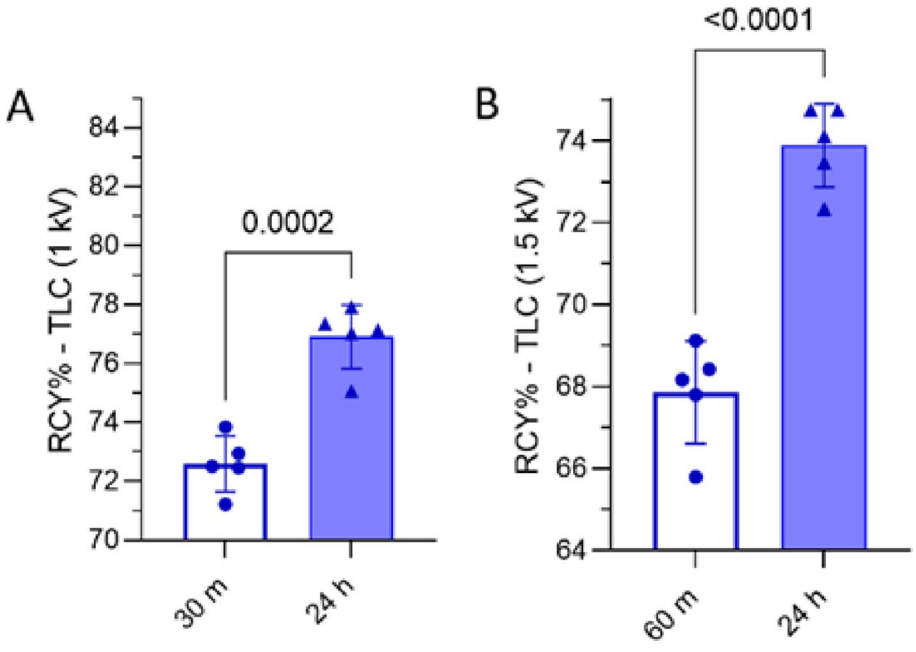

Fig. 3 Same TLC measured using **A** gas-filled proportional counter 1000 V at 30 min and 24 h; (B) 1500 V acquisition at 60 min and 24 h; both demonstrates significantly different RCY%

development. Drug products formulated with radioactive concentrations ranging from 0.851 kBq/µL to 1.702 kBq/µL (Suppl. Table 1A) were examined for each equilibrium.

Both TLC scanners were investigated for their response to francium-221 versus bismuth-213 at equilibrium (Fig. 2A, B, Suppl. Fig. 6). The gas-filled proportional counter did not show any significant differences between the alpha-specific acquisition (1000 V, 30 min post-development) versus beta/gamma-specific (1500 V at > 5 h) with a 1.2% mean difference (n = 3, P = 0.3 t-test, Fig. 2A). This demonstrated a capability to specifically discriminate alpha from beta/gamma emissions by adjusting the high voltage. This result was confirmed by comparing acquisitions at only 1000 V for the same TLC strip at 30 min and > 5 h post-development (Fig. 3A) with significant difference of RCY% (P = 0.0002, n = 5); and similarly, under 1500 V (P < 0.0001, n = 5) (Fig. 3B).

The plastic silicon detector RCY% of 61 ± 5% evaluated at 30 min post-development displayed significant differences (P < 0.0001) with 99 ± 0.1% measured at bismuth-213 equilibrium (Fig. 2B).

A TLC cut and count was applied for LSC, HPGe, and gamma well counting (Fig. 2C–E). Examining the detection at 30 min versus > 5 h post-TLC development, both LSC and gamma well counting failed to demonstrate RCY% equivalence between the two time points. Additional gamma counting of TLC measured at various energy window confirmed this observation, warranting bismuth-213 equilibrium measurements for accurate RCY% (Fig. 4). In contrast, HPGe-evaluated RCY% at 218 keV, 30 min, demonstrated exact match with that of 440 keV at > 5 h (n = 6, P = 0.9).

The RCP% evaluation using HPLC combined with gamma well counting of collected fractions showed discrepancies of 2–4% underestimation when measured at 30 min to that at > 5 h (Fig. 2F). Francium-221 secular equilibrium assessment may not adequately reflect the exact RCP% to that at bismuth-213 equilibrium. This is in line with the above observation via TLC. Also, RCY% and RCP% highly correlated within cross-instrument error at bismuth-213 secular equilibrium, complemented with HPLC–UV depicting high chemical purity with a main precursor peak, and low level of radiolytic degradations (< 3% detected) (Fig. 5, Suppl. Figs. 3, 4).

Accuracy with spiking of free actinium-225 in the drug product (DP)

DP spiking with known amounts of free actinium-225 using both TLC and HPLC methods were evaluated for accuracy (Fig. 6). The percent recovery was evaluated for each technique. The gas-filled proportional counter TLC scanners generated excellent recovery reports of expected free actinium-225 activity with 104% and 91% at 30 min (1000 V) and > 5 h (1500 V), respectively (Fig. 6A). In contrast, the silicon detector acquired at bismuth-213 secular equilibrium failed to accurately report the expected free actinium-225 (recoveries: 87%) at similar radioactive concentration (Fig. 6B). Since all free actinium-225 spikes spotted on TLC were below the LOQ of the instrument, the silicon detector failed to accurately measure actinium-225 in the DP.

Cut and count approaches using LSC, gamma well counting, and HPGe demonstrated acceptable recoveries. LSC and gamma well counting were both acquired at > 5 h post-TLC development, and resulted in 90–110% recoveries (Fig. 6C, E). HPGe demonstrated

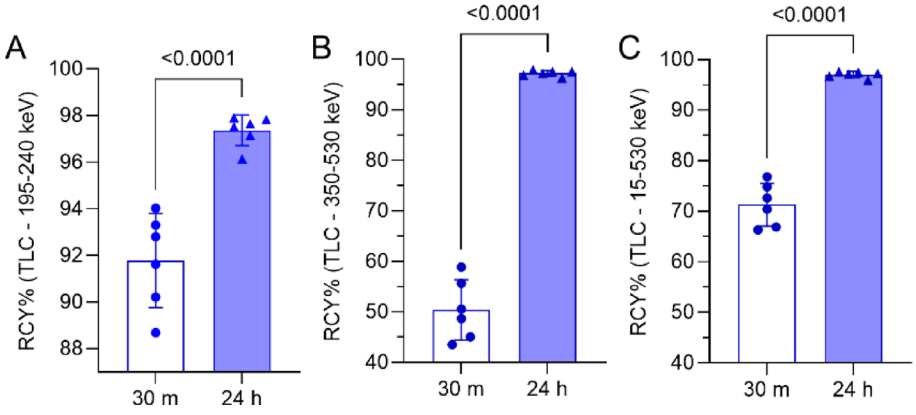

Fig. 4 Same TLC cut and measured using a gamma well counter **A** 195–240 keV at 30 min and 24 h post-TLC development; **B** 350–530 keV at 30 min and 24 h; **C** open window 15–530 keV at 30 min and 24 h. All parameters demonstrated significant RCY% differences comparing measurements of francium-221 and bismuth-213 equilibrium

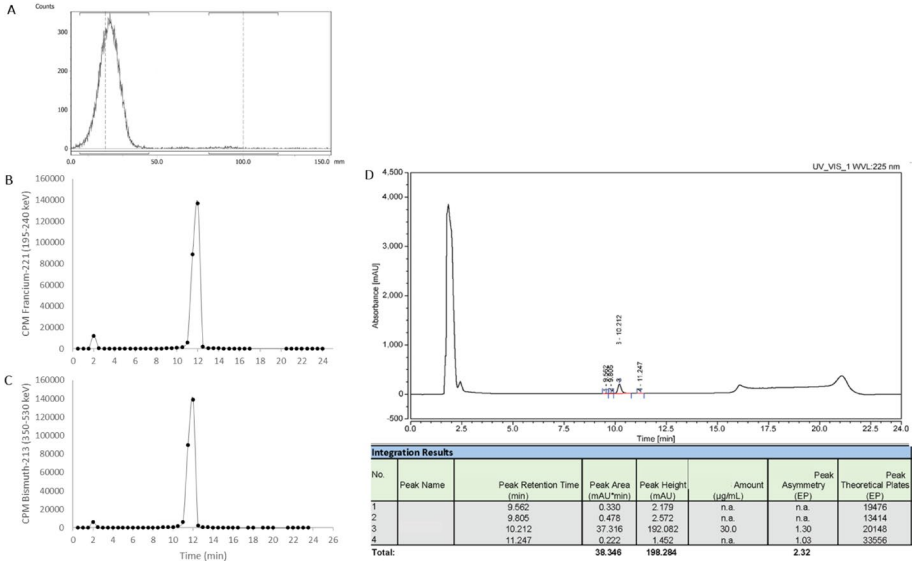

Fig. 5 TLC and HPLC chromatograms of sample 2F2 ([^{225}Ac]Ac-DOTA-peptide): **A** Silicon detector TLC scanner radiochromatogram acquired at > 5 h post-development RCY% = 98.8; **B** Fraction collected gamma counting at francium-221 window (195–240 keV) 1 h post-collection resulting in RCP% = 94.8; **C** Fraction collected gamma counting at bismuth-213 window (350–530 keV) > 5 h post-collection resulting in RCP% = 97.2; **D** matching UV-chromatogram acquired at 225 nm, chemical purity: 97.3%

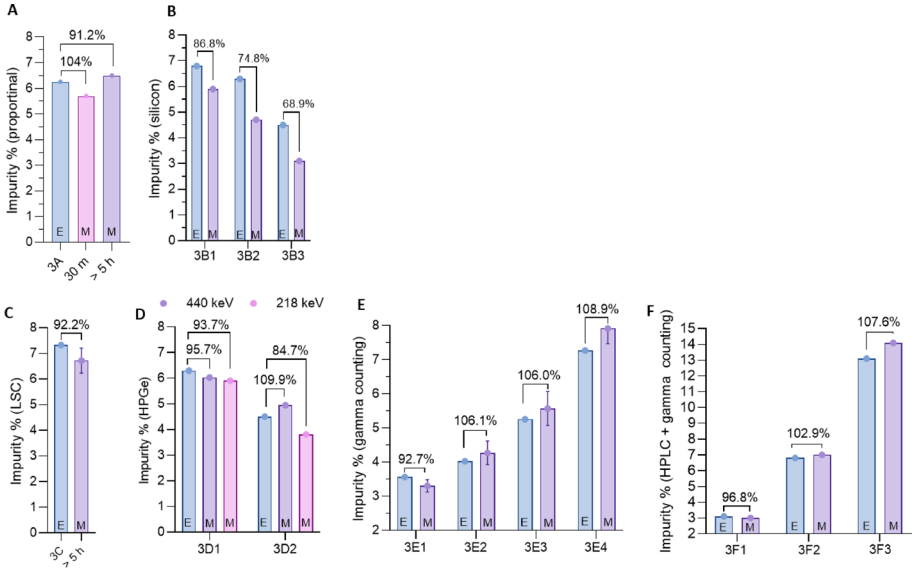

Fig. 6 Expected "E" free actinium-225 impurity % in the drug product compared with measured impurity % "M" using: TLC scanners equipped with **A** a gas-filled proportional counter at 30 min (1000 V) or > 5 h (1500 V) post-TLC development; **B** a silicon detector TLC scanner at bismuth-213 secular equilibrium. TLC cut and count measured using **C** LSC (> 5 h); **D** HPGe at 218 keV (30 min-pink) and 440 keV (> 5 h-purple); and **E** Gamma well counting (> 5 h, 350–530 keV). **F** Free actinium-225 impurity % determination using gamma well counting (350–530 keV at > 5 h) of HPLC collected fraction collection. Recoveries are reported in % on top of the columns. Sample numbering and identifiers are listed in Suppl. Table 1B

similar successful results regardless of the secular equilibrium (Fig. 6D), except for one sample nearing the LOQ (Fig. 6D-2D3).

Finally, evaluation of HPLC/gamma counted collected fractions at bismuth-213 equilibrium passed all recoveries (Fig. 6F).

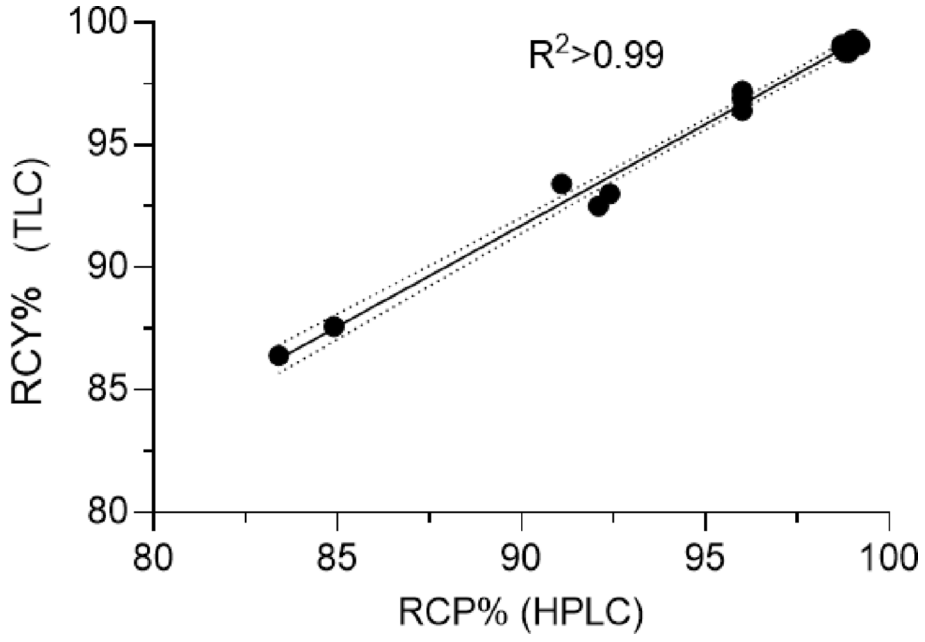

Fig. 7 Comparative RCP% vs. RCY% linear regression ($R^2 > 0.99$) of actinium-225 spiked in pure DP. The RCY% was determined using silicon detector scanner, whereas gamma counting of HPLC fraction collection was used for RCP%

Correlation of RCP% with RCY%

The [^{225}Ac]Ac-DOTA-peptide was tested for radiolytic impurities utilizing HPLC–UV and fraction collection gamma counting radio chromatograms (Fig. 5, Suppl. Figs. 3, 4, 5). Due to the low radioactive concentration of the radiopharmaceutical, the UV detection is limited by the amount of precursor injected, explaining the low intensity UV peak of the precursor. For both analytical techniques, low chemical impurities and radioimpurities were detected, with < 3% of chemical and radiochemical content. However, a difference in retention time (Rt) of 1.4 min between the original precursor UV signal at 10.1 min and the radiochromatogram peak of [^{225}Ac]Ac-DOTA-peptide at 11.5 min was observed. While the looping dead-time between the UV detector and the fraction collector was only 0.7 s, the Rt difference was explained by the analog [La]DOTA-peptide HPLC–UV (Suppl. Fig. 5A), for which the Rt was found at 11.5 min, matching the actinium-225 radiochromatogram (Fig. 5B, C and suppl. Fig. 5). The DOTA (radio)metal complexation led to a slight shift in retention time visualized for both [^{225}Ac]Ac-DOTA-peptide and its La analog.

Following the previous verifications, a number of [^{225}Ac]Ac-DOTA-peptide samples (n = 17) including free ^{225}Ac spiking assay were analyzed using both HPLC and TLC. The RCP% was then correlated to RCY%, both measured at bismuth-213 secular equilibrium. The cross-evaluation resulted in an R^2 value of > 0.99, with less than 1% difference confirming the accuracy of both techniques, and the equivalence of RCY% and RCP% (Fig. 7).

Discussion

The development of molecularly-targeted radiopharmaceutical therapy has undergone a tremendous progression over the last 10 years, launching an increasing number of first-in-human clinical trials for treatments of various cancers. In parallel, the

characterization of these drugs is not trivial with tests spanning across several expertise including chemical, radiochemical, and biological evaluations. For ^{225}Ac-radiopharmaceuticals, the path to complete QC is rather challenging due to the nature of this rare radionuclide. As of today, there is no consensus to best practices for actinium-225 QC. Actinium-225 is an alpha-emitting which decays to stability with the emission of four alpha-emitting (and two beta-emitting) progeny (Suppl. Table 2). Actinium-225 is more readily quantifiable through indirect progeny detection due to the abundance of their gamma-rays, emitted at 218 keV (francium-221) and 440 keV (bismuth-213). Historically, actinium-225 quantification has been described using bismuth-213 detection, at secular equilibrium > 5 h after separation, at this time > 98% of the bismuth-213 activity content equals that of actinium-225 (ICH guideline Q2(R2); Kelly et al. 2021 Dec 20; McDevitt et al. 2002; Maguire et al. 2014). From the standpoint of drug production manufacturing, a 5 h hold after each processing step due to equilibrium disruption is a major impediment, imposing lead times of several days for product release. Alternatively, francium-221 secular equilibrium reached after 30 min from the time of separation may offer a compelling solution to a time sensitive process.

In the present study, accuracy and precision of actinium-225 quantification, using francium-221 or bismuth-213 equilibrium, was evaluated across 5 instruments. Each instrument was first verified for linearity and LOQ detecting actinium-225 at secular equilibrium with progenies. TLC strips and HPLC fractionated samples come in different geometry. LOQ was determined for both TLC strips and solution. The LOQ measured in solution (Table 1) was found higher to that spotted on TLC strips (Table 2). A tenfold higher sensitivity was observed for TLC strips using NaI(Tl) gamma well counting and twice as high for LSC than that in solution. The difference in geometry may be the prevalent factor driving LOQ variation. Interestingly, for gamma counting, TLC spotted analysis showed almost equal LOQ (62–65 Bq) across energy windows tested; which was not the case in solution. Decreasing backscattering effect may be postulated with decreased activity measured, leading to lower LOQs.

Among all instruments tested, LSC was identified as the most sensitive technique to quantify actinium-225 with a LOQ lower than 1 Bq in solution and spotted on TLC. The RCY% determined using LSC suggested significant differences between 30 min and > 5 h acquisition, with accurate RCY% quantification only at > 5 h. The high activity spotted on the TLC strip, relative to the LSC sensitivity, contributed to quenching and poor discrimination between alpha and beta emissions (Suppl. Figs. 7, 8). As a result, LSC detection for TLC was found to be unfit to read RCY% before bismuth-213 equilibrium.

HPGe demonstrated LOQs slightly higher than LSC, or < 37 Bq. HPGe enables a high radionuclidic resolution with quantification of actinium-225 using francium-221 (218 keV, 11.4%) as early as 30 min post-TLC development, and bismuth-213 (440 keV, 25.9%) after > 5 h. This radionuclide-specific evaluation demonstrated negligible differences of RCY% at 30 min and > 5 h. HPGe was found adequate to evaluate RCY% at both equilibria.

The TLC readings using gamma counting at 195–240 keV at 30 min undervalued RCY% as compared to bismuth-213 or open readings at > 5 h (Fig. 4). Similarly, bismuth-213 readings at 350–530 keV and open window 15–530 keV measured before secular equilibrium at 30 min underestimated the true value the same way (Fig. 4B, C). Gamma well counting is known for its modest gamma resolution with high background

due to Compton scattering and the photoelectric effects in the energy range of < 200 keV. The scattering elicited by bismuth-213 has been reported to interfere with the francium-221 detection (Castillo Seoane et al. 2022). Consequently, gamma well counting is only adequate to measure actinium-225 at francium-221 equilibrium if the backscattering correction is applied for each measurement.

In contrast, the TLC scanner equipped with the gas-filled proportional counter, operated at 1000 V (alpha-specific setting), demonstrated an acceptable RCY% accuracy at 30 min, compared to measurements conducted at > 5 h at 1500 V (Fig. 2A). At 30 min post-development, the alphas emitted by actinium-225, francium-221, and astatine-217 were substantial contributors under 1000 V detection (Pretze et al. 2025). After 5 h, the equilibrated RCY% detected at 1500 V mostly capturing the beta contributions delivered by bismuth-213 and its progeny showed similar values. The measurement conducted at 1000 V after 5 h, however, did not result in accurate RCY%, confirming the specificity of this setting to alpha-emission detection (Fig. 3).

The HPLC-RCP% evaluation at francium-221 secular equilibrium, exempt of scattering correction, indicated lower accuracy compared to that of bismuth-213 equilibrium. This is mostly due to limitations of the NaI(Tl) detector to accurately quantify the francium-221 peak from background and other interferences. RCP% HPLC/gamma counting of collected fractions at francium-221 equilibrium compared to TLC-RCY% measured at bismuth-213 equilibrium showed the method was unfit for accuracy (Fig. 2F). Inter-instrument TLC and HPLC agreement was only successful when comparing RCY% and RCP% at bismuth-213 equilibrium (Fig. 7). Measuring RCP% via HPLC/gamma well counting of a ^{225}Ac-DP using francium-221 secular equilibrium result in an underestimation of 3–6% to the actual RCP%. This imprecision may result in the batch rejection of a valuable patient dose to clinic, when in fact, evaluated at bismuth-213 equilibrium the RCP% might be meeting the recommended specifications. With demonstration of radiochemical yield and purity equivalence at both equilibria, the testing may be considered as early as 30 min post-separation using TLC cut and count with HPGe or a gas proportional counter TLC scanner at 1000 V. It is of considerable value to measure RCY% this early in the process, otherwise upended by bismuth-213 equilibrium. Nonetheless, the radioimpurity quantification remains essential to define using HPLC/gamma well counting only exact at bismuth-213 secular equilibrium, due to the gamma counting limited precision at francium-221 equilibrium. For radio-degradants identification, additional assays may be considered conducting control testing of a drug product batch exempt of scavenger formulation.

In light of the detection sensitivity and LOQ defined for the gamma well counter, measurement of radio-impurities using HPLC fractions may be limited to 1% of the total content. The ^{225}Ac-DP (1.48–2.22 kBq/µL) analyzed at the end-of-synthesis, generally led to a total HPLC injection of 74–111 kBq. Detecting 0.5% impurity would require a detection of 370–555 Bq, spread across several fractions. This is at or below the gamma well counter LOQ here evaluated in solution in HPLC vial geometry (566–773 Bq in solution). When the impurity specification is raised to 1%, a minimum detection of 740 Bq to 1.11 kBq could be achieved, above the instruments LOQ. Considering the LOQ of the instrument and the low radioactive concentration of a typical ^{225}Ac-radiopharmaceutical, gamma well counting of fractionated HPLC samples may be suitable to measure only down to 1% radio-impurity. HPGe and LSC of fractions would meet 0.5%

impurity detection; however, both techniques may require labor-intensive sample transfer associated with a higher risk of operator error.

When spotting a ^{225}Ac-DP on TLC (5–10 μL), the total activity may range from 14.8 to 22.2 kBq. Detecting 0.5% of free ^{225}Ac impurity requires a TLC scanner or cut and count method to measure down to 74 Bq to 111 Bq. In light of the TLC-LOQ reported (Table 2), all techniques except the silicon detector are suitable to measure 0.5% impurity, fulfilling the recommended criteria for radiopharmaceutical QC (Gillings et al. 2020).

Linearity, accuracy, specificity and repeatability have been demonstrated utilizing 5 different instruments, all fit for actinium-225 detection when measured at bismuth-213 equilibrium. However, when using francium-221 equilibrium for actinium-225 quantification, only the HPGe and gas-filled proportional counter demonstrated adequate accuracy of RCY% ^{225}Ac-radiopharmaceutical determination.

Conclusion

Five instruments have been tested for their response to actinium-225 measurement in accuracy, sensitivity, linearity and specificity. LSC and HPGe were found to be the most sensitive techniques to detect actinium-225 progeny. Analysis and RCY% determination at francium-221 equilibrium is acceptable for HPGe and the gas-filled proportional counter using adequate parameters. LSC, gamma well counting, and the plastic silicon detector TLC scanner require bismuth-213 equilibrium for accurate RCY% characterization of the DP. Low content free actinium-225 impurity detection was accurately measured using all methods except for the silicon detector TLC scanner. Overall, this investigation sheds light on the appropriate analytical methods to deploy for ^{225}Ac-radiopharmaceuticals QC whether using francium-221 or bismuth-213 equilibria.

Abbreviations

QC	Quality control
DP	Drug product
LSC	Liquid scintillation counting
HPGe	High purity germanium
γ	Gamma counting
TLC	Thin layer chromatography
HPLC	High performance liquid chromatography
LOQ	Limit of quantification
CPM	Count per minute
RCY%	Radiochemical yield %
RCP%	Radiochemical purity %
E	Expected
M	Measured
n/a	Non assigned

Supplementary Information

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Supplementary Material 1

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Author contributions

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and editing; T. Drum: reviewing, editing, revisions and supervision; DS. Abou: conceptualization, synthesis, investigation, method development, analysis, interpretation, original draft writing, revisions and supervision.

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Data availability
All methods and data are available and reported in the manuscript or in supplemental information.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
All authors listed are full-time employees of NorthStar Medical Radioisotope., LLC; Diane Abou holds an editorial role at EJNMMI Radiopharmacy and Chemistry, Radiopharmaceutical Quality Assurance.

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