

Post-column derivatization high-performance liquid chromatography-ultraviolet-ultraviolet (HPLC-UV) method for the detection of Kryptofix 2.2.2 content in Positron Emission Tomography (PET) tracers

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ARTICLE INFO

Keywords:

Kryptofix 2.2.2

Post-column derivatization HPLC

PET tracers

Quality control

ABSTRACT

A post-column derivatization high-performance liquid chromatography-ultraviolet (HPLC-UV) method was developed to determine Kryptofix 2.2.2 (K2.2.2) in PET tracers. This method utilizes the specific complexation between K2.2.2 and Pb^{2+} , forming a stable 1:1 host-guest complex. The complexation produces a bathochromic shift in UV absorption maximum (from 210 nm to 254 nm), enabling sensitive detection. This approach addresses the limitations of poor performance and narrow applicability associated with direct single-method detection of K2.2.2.

The chromatographic conditions were optimized (column flow rate: $0.7 \text{ mL} \cdot \text{min}^{-1}$; derivatization reagent flow rate: $0.7 \text{ mL} \cdot \text{min}^{-1}$; Pb^{2+} concentration: $50 \mu\text{g} \cdot \text{mL}^{-1}$) and the method was validated. The results exhibited excellent linearity ($1\text{--}100 \mu\text{g} \cdot \text{mL}^{-1}$, $r^2 > 0.999$), high reproducibility (relative standard deviation (RSD) $< 5\%$), and a low detection limit ($0.5 \mu\text{g} \cdot \text{mL}^{-1}$). The method successfully quantified K2.2.2 in [^{18}F]FDG, [^{18}F]AV45 and [^{18}F]DPA714, achieving spiked recoveries of 89%–105%. Additionally, the method remained unaffected by interference from common sample components such as sodium chloride and sodium ascorbate.

This method provides a novel and efficient quality control tool for the simultaneous analysis of radiochemical purity and K2.2.2 content in diverse PET tracers.

1. Introduction

Positron Emission Tomography (PET) tracers, essential components of nuclear medicine imaging, employ radioactively labeled bioactive molecules targeting specific biological processes to enable dynamic visualization of physiological and biochemical processes in vivo [1]. This capability significantly enhances early diagnostic accuracy for tumors and neurodegenerative diseases [2]. Comprehensive Quality Control (QC) testing is mandatory before human administration to prevent drug-induced toxicological and pharmacological effects and their interference with PET measurements [3]. PET tracers possess distinct characteristics compared to conventional pharmaceuticals including radioactivity, short radioisotope-driven shelf-life, limited

batch sizes, and high cost per dose that make traditional testing methods inadequate for radiopharmaceutical QC [4,5]. Therefore, developing specialized QC methods and equipment is essential to minimize product loss from sample consumption during testing or radioactive decay.

Kryptofix 2.2.2 (4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo [8.8.8] hexacosane, CAS 23978-09-8, hereafter termed K2.2.2), a cryptand ligand, functions as a phase-transfer catalyst in the routine synthesis of [^{18}F]FDG (2-Deoxy-2-[^{18}F]fluoroglucose) and other ^{18}F -labeled radiopharmaceuticals to facilitate nucleophilic [^{18}F]fluorination [6–8]. Due to its significant toxicity (rat intravenous Lethal Dose, 50% (LD₅₀) = $35 \text{ mg} \cdot \text{kg}^{-1}$) [9,10], QC analysis of residual K2.2.2 in final formulations is essential. Pharmacopoeial standards including the United States Pharmacopoeia (USP), European Pharmacopoeia (EP), and Chinese

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<https://doi.org/10.1016/j.nucmedbio.2026.109610>

Received 16 November 2025; Received in revised form 24 January 2026; Accepted 20 February 2026

Available online 21 February 2026

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Pharmacopoeia specify permissible limits of K2.2.2 in [^{18}F]FDG injections [11–13].

Various analytical methods have been developed for K2.2.2 quantification in [^{18}F]FDG, including thin-layer chromatography (TLC) [14], Ultraviolet (UV) Spectrophotometry [15], gas chromatography with nitrogen-phosphorus detection (GC-NPD) [16], colorimetric spot tests [11,17], high-performance liquid chromatography (HPLC) [18], and liquid chromatography-tandem mass spectrometry (LC/MS/MS) [19]. HPLC coupled with UV detection is particularly advantageous for detecting K2.2.2 in PET tracers due to its high sensitivity, compact instrumentation, minimal sample requirements, and rapid analysis time. Notably, this technique enables simultaneous determination of radiochemical purity (RCP) and K2.2.2 levels via radio-HPLC, substantially improving QC workflow efficiency. However, the low UV absorption of K2.2.2 presents a challenge for direct HPLC detection, frequently resulting in interference and reduced result reliability.

K2.2.2 possesses a unique 3D cavity structure (diameter = 2.8 Å [20]) that selectively complexes metal ions through ion-dipole interactions. When the host cavity and guest ion demonstrate geometric size matching (e.g., with Pb^{2+}) diameter = 2.4 Å [21]), it forms stoichiometrically stable 1:1 host-guest complexes [22–24]. This complexation produces a bathochromic shift in UV absorption, allowing sensitive detection at 254 nm. Zhao [25] developed a method for detecting K2.2.2 in [^{18}F]FDG using K2.2.2- Pb^{2+} complex via UV detection. This approach is minimally influenced by temperature, achieves maximum absorbance rapidly under ambient conditions, and produces a stable product suitable for long-term storage. However, the absorption peak of the K2.2.2- Pb^{2+} complex may coincide with those of primary products from other PET tracers. This limitation constrains the method's applicability to select PET tracers such as [^{18}F]AV45 (4-[(E)-2-(6-{2-[2-(^{18}F)fluoroethoxy]ethoxy}-3-pyridinyl)vinyl]-N-methylaniline) and [^{18}F]DPA714 (N,N-diethyl-2-[4-(2-(^{18}F)fluoroethoxy)phenyl]-5,7-dimethylpyrazolo[1,5-a]pyrimidine-3-acetamide), which have characteristic absorption at 254 nm.

We developed a post-column derivatization HPLC-UV method to quantify K2.2.2- Pb^{2+} complexes in PET samples. This approach utilizes the separation power of HPLC to isolate K2.2.2 from the sample matrix before the chemical transformation (derivatization) with Pb^{2+} . By separating the chromatographic separation of K2.2.2 from subsequent Pb^{2+} complexation, this approach maintains high analytical sensitivity while achieving superior method specificity. Its efficient workflow and structural versatility provide a novel, interference-free QC method for structurally diverse PET tracers.

2. Materials and methods

2.1. Materials

The K2.2.2 standard was purchased from ABX GmbH (Radeberg, Germany). HPLC-grade acetonitrile and trifluoroacetic acid (TFA) were obtained from Aladdin (Shanghai, China). Unless otherwise specified, all other chemicals were of analytical grade. Stock solutions of K2.2.2 and lead nitrate ($\text{Pb}(\text{NO}_3)_2$) were prepared in water at a concentration of 1000 $\mu\text{g}\cdot\text{mL}^{-1}$, and subsequently diluted with water to the desired concentrations. All aqueous solutions were prepared using 18.2 M Ω deionized water from a Heal Force water purification system (Shanghai, China). All HPLC mobile phases were degassed by sonication prior to use. Samples of [^{18}F]AV45 and [^{18}F]DPA714 were synthesized using the OnePlatform® 3.2 multi-functional automatic synthesis module (JIRUI TECH, Co., Ltd., Hangzhou, China) in our laboratory. Synthesized tracers were allowed to decay for at least 20 half-lives before testing to ensure non-radioactive samples for UV analysis.

2.2. Equipment

Formation of the K2.2.2- Pb^{2+} complex and its UV absorption

characteristics were analyzed using a fiber optic spectrometer (SEEMAN Co., Ltd., Hangzhou, China) equipped with an autosampler system featuring a 50 μL flow cell. The spectrophotometer operated at a flow rate of 1 $\text{mL}\cdot\text{min}^{-1}$. The light source wavelength range was 190–800 nm, with auto-exposure enabled. Prior to use, the instrument underwent manual wavelength calibration using the characteristic emission lines of the deuterium lamp. The post-calibration wavelength error was $\leq \pm 0.2$ nm.

The quantification of K2.2.2 in [^{18}F]FDG samples was conducted using a MAPADA P3 UV spectrophotometer (Shanghai, China), equipped with a quartz cuvette featuring a 10 mm optical path length and 1 mL capacity.

Chromatographic separation was conducted using an in-house developed automated miniature HPLC system (Fig. 1), equipped with a high-pressure liquid chromatography pump, a 20 μL manual injection valve, a degassing module, and a post-column derivatization module. For the simultaneous analysis of K2.2.2 and RCP in [^{18}F]FDG, a Phenomenex Luna Omega Sugar column (4.6 mm i.d. $\times$ 150 mm, 5.0 μm) was utilized. For all other analyses, a GL Sciences Inertsil ODS-3 column (4.6 mm i.d. $\times$ 250 mm, 5.0 μm) was employed. Column effluent was monitored simultaneously at 210 nm and 254 nm using an ECOM DAD400 UV/VIS detector (Chrastany, Czech Republic). Data acquisition and processing were performed using Tangram control software (Orienda Co., Ltd., Beijing, China). Post-column derivatization was conducted at ambient temperature using an identical model high-pressure liquid pump and a T-junction mixer (The total mixing volume from the point where the mobile phase and derivatization solution combine at the T-junction mixer to the inlet of the UV detector is approximately 500 μL).

2.3. Methods

2.3.1. Fiber optic spectrophotometer analysis

The fiber optic spectrophotometer was initialized for 30 min prior to testing to ensure light source stabilization. System calibration was performed using pure water before each test session.

The complex for spectral testing was prepared by thoroughly combining a 25 $\mu\text{g}\cdot\text{mL}^{-1}$ K2.2.2 standard solution and a 50 $\mu\text{g}\cdot\text{mL}^{-1}$ Pb standard solution ($\text{Pb}(\text{NO}_3)_2$). Its spectrum was analyzed against those of the individual components to determine the maximum absorption wavelength within the range of 200–800 nm.

To examine the effect of pH on complex stability, 2.5 mL of a 1000 $\mu\text{g}\cdot\text{mL}^{-1}$ K2.2.2 standard solution and 5 mL of a 1000 $\mu\text{g}\cdot\text{mL}^{-1}$ Pb standard solution were combined thoroughly and added to 92.5 mL of aqueous solutions at various pH values. Spectrophotometric analysis commenced immediately. The response at the identified maximum wavelength was monitored continuously to assess the impact of different pH levels on complex stability. System calibration for these tests utilized a mixture of 7.5 mL pure water and 92.5 mL of the corresponding pH solution, performed before each pH group measurement.

2.3.2. UV spectrophotometer analysis

The UV spectrophotometer was initialized for 30 min prior to testing to ensure system stabilization.

UV spectrophotometry was employed to quantify K2.2.2 in actual [^{18}F]FDG samples. Precisely measured volumes (0.00, 0.05, 0.10, 0.20, 0.40 mL) of a 100 $\mu\text{g}\cdot\text{mL}^{-1}$ K2.2.2 standard solution were placed into separate 5 mL EP tubes (Eppendorf tubes). To each tube, 1.0 mL of pH 6.4 citric acid-sodium hydroxide buffer solution (prepared by dissolving 5.25 g citric acid and 2.0 g NaOH in 50 mL water, adjusting to pH 6.4 using 0.1 $\text{mol}\cdot\text{L}^{-1}$ NaOH and a pH meter, and diluting to 250 mL) and 1.0 mL of a 500 $\mu\text{g}\cdot\text{mL}^{-1}$ Pb lead nitrate solution (prepared by dissolving 79.93 mg $\text{Pb}(\text{NO}_3)_2$ in water and diluting to 100 mL) were added. The volume in each tube was then adjusted to 4 mL with water and mixed thoroughly. Absorbance was measured at 253 nm in order of increasing concentration to construct a calibration curve. Method precision was

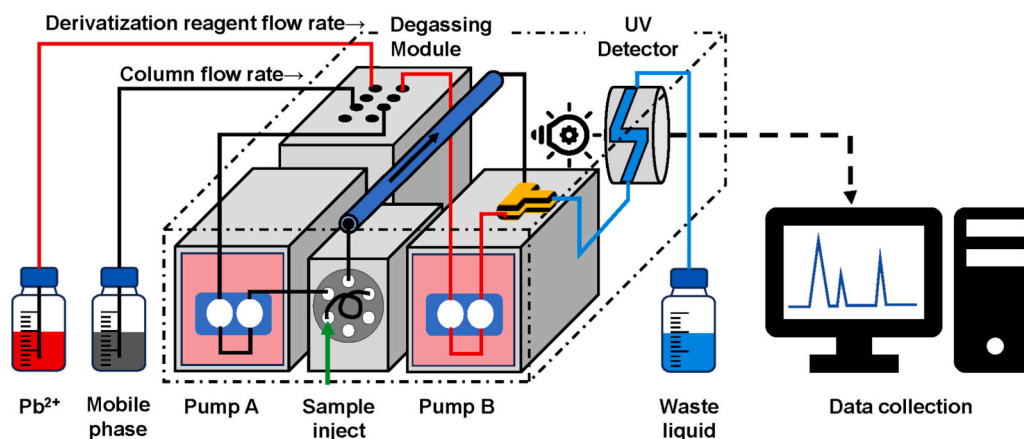


Fig. 1. Description of the post-column derivatization system for HPLC.

evaluated using the relative standard deviation (RSD) of six consecutive measurements of the standard sample corresponding to the 0.05 mL addition point on the calibration curve. The Limit of Detection (LOD) was determined by measuring a blank sample six times and calculating 3 times the standard deviation at the 95% confidence level. The K2.2.2 content in original [^{18}F]FDG samples was measured. Additionally, spiked samples were prepared by adding 0.1 mL of the 100 $\mu\text{g}\cdot\text{mL}^{-1}$ K2.2.2 standard solution to the original samples. Spike recovery was calculated using the original and spiked samples.

The lead-containing waste generated during experiments was primarily treated by solid-phase extraction (SPE) using a thiol-based adsorbent to effectively concentrate and remove Pb^{2+} . This waste treatment strategy is a critical component for the safe implementation of the method (see Section 3.5).

2.3.3. HPLC analysis

The HPLC mobile phase consisted of acetonitrile-50 mM ammonium acetate (1:1, v/v), premixed before use. Isocratic elution was conducted at room temperature with manual injection of 20 μL . The optimization of column flow rate, derivatization reagent flow rate, and derivatization reagent concentration was performed using a concentration equivalent to four times the standard specified limit of K2.2.2 (i.e., 100 $\mu\text{g}\cdot\text{mL}^{-1}$) to facilitate more sensitive observation [12,13].

Method linearity was evaluated using K2.2.2 standard solutions at concentrations of 1, 5, 10, 20, 50, and 100 $\mu\text{g}\cdot\text{mL}^{-1}$. Samples were injected in order of increasing concentration, with duplicate injections per level. Peak areas were averaged, and linearity with the correlation coefficient (r^2) was determined using the least-squares method. Method precision (Repeatability) was assessed through six consecutive injections of a 5 $\mu\text{g}\cdot\text{mL}^{-1}$ K2.2.2 standard solution and calculating the RSD of the target peak areas. The method detection limit was determined as the lowest detectable concentration, defined as a signal-to-noise ratio (SNR) of 3. The K2.2.2 content was measured in original samples of [^{18}F]FDG, [^{18}F]AV45 and [^{18}F]DPA714. Additionally, spiked samples containing 10 $\mu\text{g}\cdot\text{mL}^{-1}$ K2.2.2 were prepared for each tracer and injected in duplicate. Spike recovery was calculated to evaluate the method's practical application.

2.3.4. Simultaneous detection of RCP and K2.2.2

Method Validation for [^{18}F]FDG: The mobile phase consisted of acetonitrile-water (85:15, v/v), premixed before use. Isocratic elution was conducted at room temperature with a manual injection volume of 20 μL . The column flow rate and derivatization reagent flow rate were both maintained at 0.7 $\text{mL}\cdot\text{min}^{-1}$, with the derivatization reagent containing Pb^{2+} at 50 $\mu\text{g}\cdot\text{mL}^{-1}$. The method's performance was evaluated by: (1) comparing chromatograms of a 100 $\mu\text{g}\cdot\text{mL}^{-1}$ K2.2.2 standard solution before and after derivatization, (2) testing with 0.9% (w/v)

sodium chloride solution, and (3) assessing its applicability to actual [^{18}F]FDG samples.

Method validation for other PET tracers: The mobile phase comprised acetonitrile and water containing 0.05% (v/v) TFA, which was pre-mixed prior to use. Isocratic elution was performed at room temperature with a manual injection volume of 20 μL . The column flow rate and the derivatization reagent flow rate were both maintained at 0.7 mL/min . The concentration of Pb^{2+} in the derivatization reagent was 50 $\mu\text{g}\cdot\text{mL}^{-1}$. A standard solution of the K2.2.2- Pb^{2+} complex was prepared by combining 90 $\mu\text{g}\cdot\text{mL}^{-1}$ K2.2.2 and 50 $\mu\text{g}\cdot\text{mL}^{-1}$ Pb^{2+} . Following thorough mixing, 20 μL was manually injected to evaluate the complex's stability under acidic mobile phase conditions.

2.3.5. Data analysis

MATLAB was utilized for generating graphs. All HPLC chromatograms underwent baseline correction prior to plotting. Peak area data were acquired automatically by the data collection software and processed using Microsoft Excel.

3. Results and discussion

3.1. Spectral analysis of K2.2.2- Pb^{2+} complex

Initially, the investigation focused on the effect of Pb^{2+} on the spectral properties of K2.2.2 complexation. The addition of Pb^{2+} enhanced UV absorption by K2.2.2 and shifted its characteristic absorption peak from 210 nm to 254 nm. Fig. 2A illustrates the characteristic absorption peaks and intensities for K2.2.2, Pb^{2+} , and K2.2.2- Pb^{2+} complex. Complexation with Pb^{2+} substantially enhanced detection sensitivity compared to K2.2.2 alone. Furthermore, the absorption peak shift reduced interference from UV absorption of other substances, improving the method's applicability.

HPLC analysis of K2.2.2 traditionally employs a mobile phase of 50 mM ammonium acetate-acetonitrile (1:1, v/v) on a C18 stationary phase column. Building on this approach, the complexation reaction between Pb and K2.2.2 was incorporated into the HPLC system to examine differences between pre-column and post-column derivatization of K2.2.2. These methods were compared against direct injection of K2.2.2. As shown in Fig. 2B, the pre-column derivatization method demonstrated significant peak tailing, without substantial improvement in detection sensitivity compared to direct injection. Conversely, the post-column derivatization method produced normal peak shapes and achieved at least a 3-fold increase in detection sensitivity at equivalent concentrations.

Spectral and chromatographic analysis of K2.2.2 and the K2.2.2- Pb^{2+} complex demonstrated that post-column derivatization with Pb effectively detects K2.2.2. This finding supports the development of

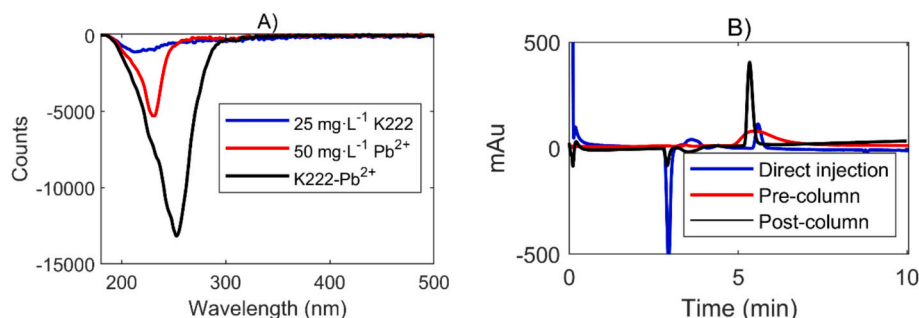


Fig. 2. A) UV absorption spectra of K2.2.2, Pb²⁺, and the K2.2.2-Pb²⁺ complex. B) HPLC profile comparison: K2.2.2-Pb²⁺ complex pre-column derivatization vs. post-column derivatization vs. direct K2.2.2 injection (mobile phase: 50 mM ammonium acetate-acetonitrile (1:1, v/v); column flow rate: 0.7 mL·min⁻¹; K2.2.2 concentration: 100 µg·mL⁻¹).

improved QC methods for PET tracers, offering the necessary methodological versatility to be applied reliably to a wide range of structurally diverse radiopharmaceuticals.

3.2. Optimization of post-column derivatization conditions

The chromatographic performance of K2.2.2 was evaluated at column flow rates ranging from 0.5 to 1.0 mL·min⁻¹. Fig. 3A demonstrates the relationship between sensitivity and column flow rate. The sensitivity of K2.2.2 within the retention time window initially increased and subsequently decreased as the flow rate decreased, reaching its maximum at 0.7 mL·min⁻¹. Flow rates below 0.7 mL·min⁻¹ resulted in shoulder peak formation and diminished resolution. Based on these considerations of sensitivity and resolution, a flow rate of 0.7 mL·min⁻¹ was determined optimal.

Derivatization reagent flow rate optimization was conducted at a fixed column flow rate of 0.7 mL·min⁻¹, as illustrated in Fig. 3B. The derivatization reagent flow rate predominantly affected the sample

response intensity and baseline noise level. Higher flow rates of the derivatization reagent corresponded to lower target analyte responses, primarily due to dilution effects. Additionally, the relationship between derivatization reagent flow rate and column flow rate affected baseline noise intensity. The optimal derivatization reagent flow rate (0.7 mL·min⁻¹) was selected based on the highest SNR.

The post-column derivatization HPLC system utilized in this research was not specifically designed for baseline noise reduction. Therefore, a 1:1 volumetric ratio of mobile phase to derivatization reagent was implemented for post-column mixing to optimize stability and sensitivity. In HPLC-post-column derivatization detection systems specifically engineered for low noise operation, reduced derivatization reagent flow rates might be feasible to minimize signal dilution effects.

The impact of derivatization reagent (Pb²⁺) concentration on method detection sensitivity was examined by analyzing K2.2.2 across a derivatization solution concentration range of 10–200 µg·mL⁻¹, as shown in Fig. 3C. The derivatization reagent concentration influenced detection sensitivity in two primary ways: increasing reagent

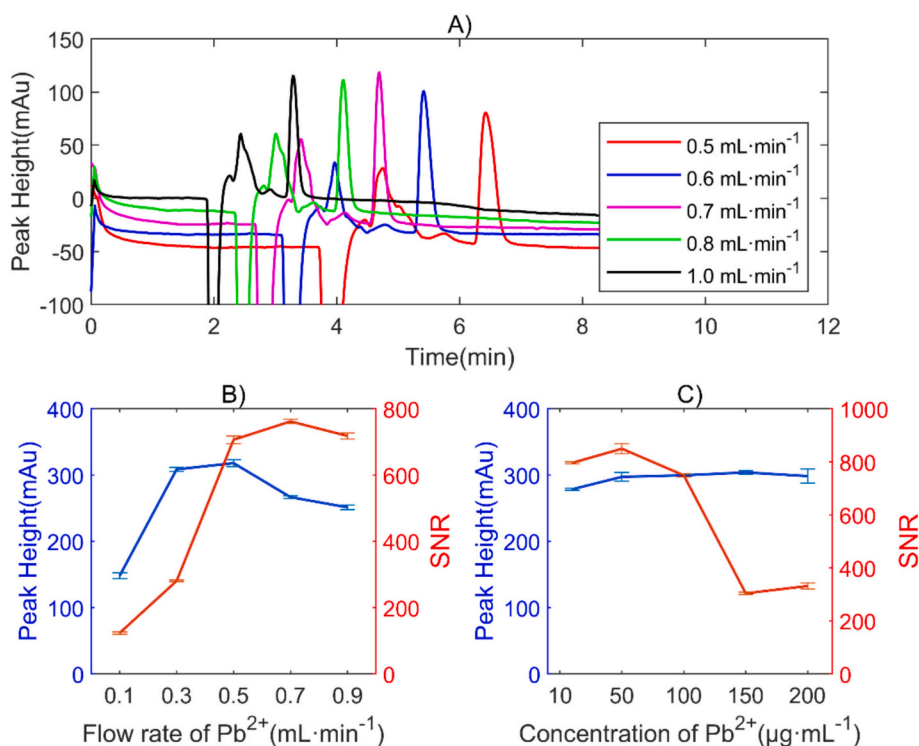


Fig. 3. HPLC detection results of the K2.2.2-Pb²⁺ complex under different conditions. A) Various column flow rates (derivatization reagent concentration: 100 µg·mL⁻¹). B) Various derivatization reagent flow rates (derivatization reagent concentration: 100 µg·mL⁻¹, column flow rate: 0.7 mL·min⁻¹). C) Various derivatization reagent concentrations (column and derivatization reagent flow rates: 0.7 mL·min⁻¹).

concentration led to maximization of derivatization product content and gradual increase in target peak height until stabilization. However, excessive reagent concentrations resulted in increased system noise, thereby reducing detection sensitivity. Based on comprehensive analysis of detection sensitivity, 50 $\mu\text{g}\cdot\text{mL}^{-1}$ was established as the optimal derivatization concentration.

3.3. Method performance

The analytical performance of UV and HPLC with post-column derivatization for K2.2.2 detection was evaluated comparatively. Table 1 presents the detection wavelength, analytical dynamic range, RSD, and LOD for each method.

Method reproducibility was assessed through analysis of a standard K2.2.2 solution (5 μg for UV, 20 μL of 5 $\mu\text{g}\cdot\text{mL}^{-1}$ for HPLC). Both methods demonstrated RSD values within 5% ($n = 6$).

The UV method exhibited linearity across 0–80 $\mu\text{g}\cdot\text{mL}^{-1}$, with a correlation coefficient (r^2) exceeding 0.998. The HPLC method, using a 20 μL injection volume, demonstrated linear detector response for the K2.2.2-Pb²⁺ complex from 0 to 100 $\mu\text{g}\cdot\text{mL}^{-1}$, with correlation coefficients (r^2) greater than 0.999.

The limit of detection (LOD) for the UV method, calculated as 3σ (where σ represents the standard deviation of the blank), was determined to be 0.972 $\mu\text{g}\cdot\text{mL}^{-1}$. The LOD (signal-to-noise ratio = 3) for the HPLC method with a 20 μL injection volume was 0.5 $\mu\text{g}\cdot\text{mL}^{-1}$.

The developed method (HPLC with post-column derivatization) was applied to analyze K2.2.2 in [¹⁸F]FDG, [¹⁸F]AV45, and [¹⁸F]DPA714 produced in our laboratory (representative chromatograms shown in Fig. 4). The method's accuracy was determined by analyzing samples spiked with a 10 $\mu\text{g}\cdot\text{mL}^{-1}$ K2.2.2 standard mixture. The results were compared with those obtained through ultraviolet spectrophotometry for K2.2.2 analysis in [¹⁸F]FDG. The quantification of K2.2.2 in both pristine PET samples and spiked samples was conducted using the linear regression equations provided in Table 1, with results presented in Table 2.

Recoveries ranged from 89.14% to 103.04%, indicating that the proposed method is suitable for QC analysis of K2.2.2 in [¹⁸F]AV45 and [¹⁸F]DPA714 formulations. For the same batch of [¹⁸F]FDG, the HPLC post-column derivatization method yielded more precise quantification of K2.2.2 content compared to spectrophotometric analysis. The K2.2.2 levels in all tested samples satisfied the acceptance criteria (<25 $\mu\text{g}\cdot\text{mL}^{-1}$) specified in both USP and EP for radiopharmaceutical QC [12,13].

Literature and experimental results confirm that several common components of PET radiopharmaceutical formulations do not interfere with the quantification of K2.2.2. These include sodium ascorbate (40 mM, pH 5.6), sodium chloride (0.9% w/v), and excess precursors from the synthesis of [¹⁸F]FDG, [¹⁸F]AV45, and [¹⁸F]DPA714 [26,27].

Table 1

Comparison of reproducibility, linearity, and detection limit for the analysis of the K2.2.2-Pb²⁺ complex: post-column derivatization combined with ultraviolet spectrophotometry vs. direct ultraviolet spectrophotometry.

Method	Linear range ($\mu\text{g}\cdot\text{mL}^{-1}$)	Linear regression equation ($\mu\text{g}\cdot\text{mL}^{-1}$, [r^2])	Reproducibility RSD (%)	Detection limit ($\mu\text{g}\cdot\text{mL}^{-1}$)
Post-column derivatization HPLC	1–100	$y = 0.708x - 0.701$, [0.999]	4.13	0.5
UV	0–80	$y = 0.0033x$, [0.998]	3.603	0.972

3.4. Simultaneous analysis of PET tracer K2.2.2 and RCP

To evaluate the potential for simultaneous post-column derivatization HPLC analysis of K2.2.2 using Pb²⁺ alongside RCP testing of PET tracers, the mobile phases commonly used in RCP analysis were reviewed from the literature and compared with RCP testing conditions utilized in our laboratory for PET tracer analysis [7,8,27–31]. Three primary RCP analysis conditions were identified: acetonitrile-acetate, acetonitrile-water, and acetonitrile-TFA aqueous systems.

3.4.1. Application of the acetonitrile-ammonium acetate mobile phase

The mobile phase utilized in this investigation, 50 mM ammonium acetate-acetonitrile, aligns with previously reported conditions for [¹⁸F]AV45 and [¹⁸F]DPA714. Specifically, analysis of [¹⁸F]AV45 utilized an ammonium acetate-acetonitrile mobile phase (11:9, v/v) at a flow rate of 1 $\text{mL}\cdot\text{min}^{-1}$ on a reversed-phase C18 column, with the radioactive peak of the target compound eluting within a retention time (RT) range of 5.5–6.5 min [27]. For [¹⁸F]DPA714, the mobile phase consisted of ammonium acetate-acetonitrile (6:4, v/v) under the same flow rate and column conditions, yielding a radioactive peak between 8.5 and 9.5 min RT [26]. In the present study, using an ammonium acetate-acetonitrile (1:1, v/v) mobile phase at 1 $\text{mL}\cdot\text{min}^{-1}$ on a reversed-phase C18 column, the K2.2.2 target peak was detected within an RT range of 3–4 min (see Fig. 3A), demonstrating the feasibility of simultaneous detection. Representative chromatograms for the simultaneous detection of K2.2.2 and RCP in the PET tracers [¹⁸F]AV45 and [¹⁸F]DPA714 are shown in Fig. 5.

3.4.2. Application of the acetonitrile-water mobile phase

The RCP analysis for [¹⁸F]FDG was performed using a Luna Omega sugar column (4.6 mm $\times$ 150 mm, 5.0 μm) with an acetonitrile-water (85:15, v/v) mobile phase at 1 $\text{mL}\cdot\text{min}^{-1}$. To address the potential risk of interference, we critically evaluated two key aspects. First, UV detection at 254 nm confirmed that neither [¹⁸F]FDG nor its precursors exhibit any detectable absorption, eliminating spectral interference. Second, and most critically, the absence of absorption confirms that [¹⁸F]FDG does not undergo derivatization with Pb²⁺, as such a reaction would require the formation of a UV-absorbing complex. This was further corroborated by the clean baseline at the retention time of the K2.2.2-Pb²⁺ complex (RT $\approx$ 2 min) when analyzing [¹⁸F]FDG samples. Furthermore, the method demonstrated specificity as the K2.2.2 peak (RT $\approx$ 2 min) was well-resolved from the radioactive [¹⁸F]FDG product peak (RT $\approx$ 4.5 min), and the presence of 0.9% sodium chloride did not cause interference (Fig. 6).

3.4.3. Application of the acetonitrile-TFA water mobile phase

TFA, characterized by strong acidity ($\text{pK}_a = 0.23$), functions primarily as an ion-pairing reagent in HPLC mobile phases, facilitating pH adjustment, peak shape improvement, and enhanced separation efficiency. Based on established RCP testing conditions in our laboratory, mobile phases containing varying TFA proportions effectively meet RCP analysis requirements for most PET tracers, excluding [¹⁸F]FDG. Consequently, the application of the post-column derivatization method in TFA-containing mobile phases was examined.

The influence of pH on complex stability was assessed by monitoring the spectral intensity of the complex solution temporally under various pH conditions. As illustrated in Fig. 7, complex intensity varied with time at different pH values. In environments below pH 3.8, the complex exhibited gradual degradation, with degradation rates correlating positively with solution acidity. At pH $\geq$ 3.8, the complex maintained stability for periods exceeding 30 min.

An aqueous solution containing 0.05% TFA has a pH < 3, creating unfavorable conditions for stable complex formation. Given TFA's low ionization degree in pure acetonitrile ($\text{pK}_a = 12.65$ in acetonitrile [32]), acetonitrile addition suppresses TFA dissociation in water, potentially providing suitable pH conditions for post-column derivatization. This

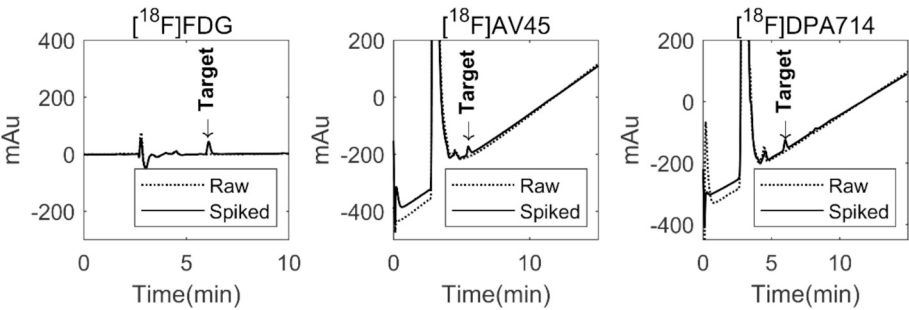


Fig. 4. Chromatograms of K2.2.2 spiking test in PET tracer (mobile phase: 50 mM ammonium acetate-acetonitrile (1:1, v/v); column and derivatization reagent flow rates: 0.7 mL·min⁻¹; derivatization reagent concentration: 50 μg·mL⁻¹)

Table 2
Comparison of recovery rates for the K2.2.2-Pb²⁺ complex: post-column derivatization combined with ultraviolet spectrophotometry vs. direct ultraviolet spectrophotometry.

Method	Compound	Quantity (μg·mL ⁻¹)	Accuracy		
			Added (μg·mL ⁻¹)	Found (μg·mL ⁻¹)	Recovery (%)
Post-column derivatization HPLC	[¹⁸ F]FDG	1.18 ± 0.06	10	11.49 ± 0.05	103.04
	[¹⁸ F]AV45	<0.5	10	8.91 ± 0.16	89.14
	[¹⁸ F]DPA714	<0.5	10	9.34 ± 0.13	93.45
UV	[¹⁸ F]FDG	8.79 ± 0	20	29.7 ± 0	104.55

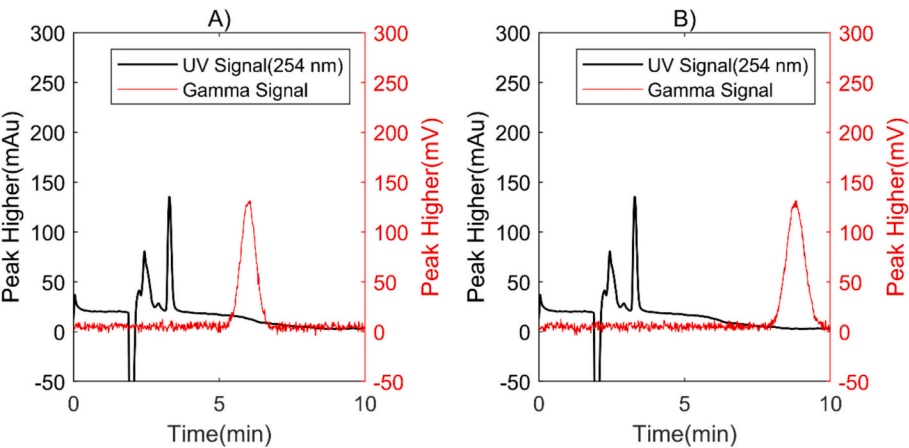


Fig. 5. Chromatogram of K2.2.2 and RCP. A) Chromatogram of [¹⁸F]AV45. B) Chromatogram of [¹⁸F]DPA714. (Radio-HPLC chromatogram of the PET tracer (schematic from literature) demonstrating the feasibility of simultaneous detection of K222 and RCP.)

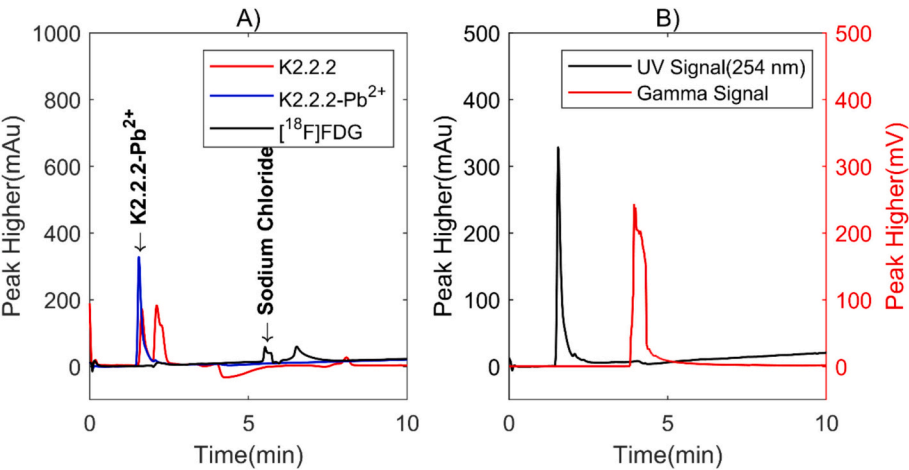


Fig. 6. Chromatogram of K2.2.2: acetonitrile-water (85:15, v/v). A) Single-component test chromatogram, B) Simultaneous analysis of K2.2.2 and RCP in [¹⁸F]FDG

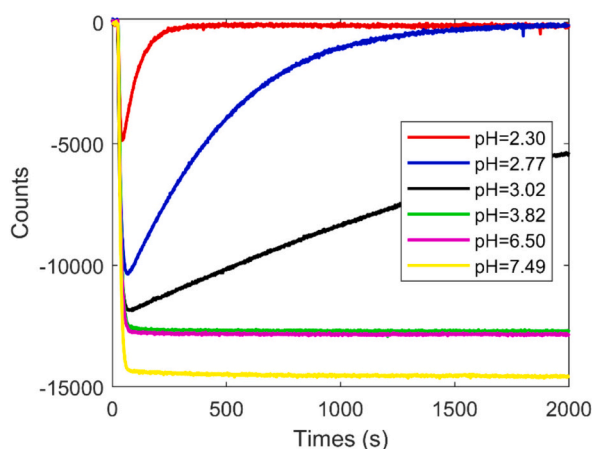


Fig. 7. Stability differences of K2.2.2-Pb²⁺ complexes at different pH values.

was verified by direct injection of 20 μL K2.2.2-Pb²⁺ standard solution under mobile phase conditions with varying acetonitrile ratios, as shown in Fig. 8. Higher acetonitrile proportions in the mobile phase resulted in more neutral pH values, with minimal complex response loss during chromatographic separation at acetonitrile contents $\geq 20\%$.

During practical application in post-column derivatization, observations revealed that a higher mobile phase pH (0.05% TFA: acetonitrile (3:7, v/v), pH $\approx$ 6) did not enhance the complexation reaction under these conditions. This result suggests that the high organic content itself, or residual interactions with TFA, directly interferes with the K2.2.2-Pb²⁺ binding kinetics in the dynamic reactor. This observation differs from the findings reported by Zhao [25]. Although the complex maintained stability in this environment, the complexation reaction was inhibited. The addition of acetonitrile showed minimal improvement in facilitating the complexation reaction under acidic conditions.

The paper concludes that the complex is unstable at a pH below 3.8 and that the pH would need to be adjusted before derivatization in acidic environments. The most practical approach for post-column pH adjustment in acidic environments is to utilize a third reagent stream (a third HPLC pump). This stream should contain a concentrated buffer (such as ammonium acetate or NaOH) added via a T-mixer, prior to the introduction of Pb²⁺. Optimization of the flow rate and concentration is essential to quickly neutralize the column effluent to a stable pH (ideally between 6 and 7), ensuring the immediate and stable formation of the

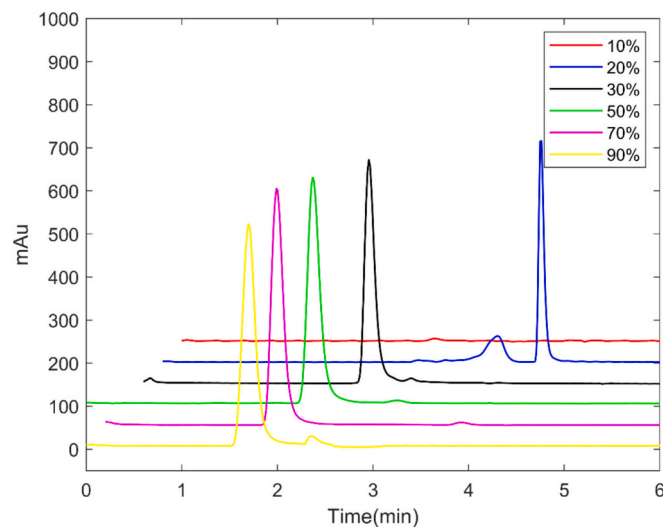


Fig. 8. Chromatogram of K2.2.2-Pb²⁺ complex under mobile phases with different acetonitrile ratios.

K2.2.2-Pb²⁺ complex while minimizing peak dilution.

Furthermore, as shown in Fig. 8, compared to the neutral mobile phase composition of acetonitrile-ammonium acetate, the K2.2.2-Pb²⁺ complex under acidic mobile phase conditions exhibited favorable peak shape and response during on-column injection. This is likely because the hydrogen ions in the acidic mobile phase suppress competitive binding from other ions, thereby maintaining the stability of the complex. This characteristic of the K2.2.2-Pb²⁺ complex provides a novel approach for the sensitive detection of K2.2.2 in acidic environments. However, the presence of saline in PET tracers may introduce interference, potentially affecting the accuracy of the results.

3.5. Practical considerations for clinical implementation

While the proposed post-column derivatization method offers significant advantages in sensitivity and specificity, its practical implementation in a Good Manufacturing Practice (GMP)-compliant clinical radiopharmacy setting warrants careful consideration, primarily due to the use of toxic Pb²⁺ salts.

To prevent potential cross-contamination of other analytical systems or samples with lead, it is strongly recommended that a dedicated HPLC system, or at minimum a dedicated fluidic path (including the derivatization pump, mixing tee, connecting tubing, and UV flow cell), be employed exclusively for K2.2.2 analysis. This precautionary measure is essential to maintain the integrity of other quality control tests and to comply with stringent GMP requirements regarding contamination control.

Regarding waste generation, under the optimized conditions described in this study (column flow rate: 0.7 mL·min⁻¹; derivatization reagent flow rate: 0.7 mL·min⁻¹; derivatization concentration: 50 $\mu\text{g}\cdot\text{mL}^{-1}$; typical run time including equilibration: ~ 15 min), each analytical run generates approximately 21 mL of Pb²⁺-containing waste with a concentration of 25 $\mu\text{g}\cdot\text{mL}^{-1}$. This volume is manageable within a standard radiopharmaceutical laboratory's waste handling protocols. As mentioned in Section 2.3.2, all lead-containing waste should be treated by SPE using a thiol-based adsorbent to effectively concentrate and remove Pb²⁺ prior to final disposal. Implementing this on-line or off-line SPE step as part of the standard operating procedure is crucial for safe and compliant waste management.

4. Conclusion

This study established a post-column derivatization HPLC-UV method for quantitative K2.2.2 analysis in PET tracers, based on the highly selective complexation reaction between K2.2.2 and Pb²⁺. The method exhibited good linearity within 1–100 $\mu\text{g}\cdot\text{mL}^{-1}$, demonstrating enhanced method stability and detection limits compared to UV spectrophotometry. Analysis of identical [¹⁸F]FDG sample batches yielded K2.2.2 concentrations of $1.18 \pm 0.06 \mu\text{g}\cdot\text{mL}^{-1}$ using this method, showing significantly greater precision (e.g., lower RSD) than the 8.79 $\mu\text{g}\cdot\text{mL}^{-1}$ obtained via UV spectrophotometry. The method's utility extends beyond [¹⁸F]FDG to other PET tracers, achieving practical spike recovery rates between 89% and 105%. Crucially, this methodology overcomes the matrix interference and low sensitivity inherent to direct UV methods. It enables simultaneous detection of K2.2.2 and RCP in various PET tracers within acetonitrile-water and acetonitrile-ammonium acetate environments, thus improving quality control efficiency and reducing sample consumption.

This methodology presents a viable alternative to conventional TLC, with reduced equipment costs and maintenance requirements. Consequently, the developed post-column HPLC-UV method serves as a reliable, highly precise, and efficient solution for rapid PET tracer QC in GMP-compliant radio-pharmaceutical production.

CRediT authorship contribution statement

Lei Xu: Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Zhenhao Dong:** Writing – original draft, Methodology, Investigation. **Hong Zhang:** Supervision, Resources, Project administration, Methodology, Funding acquisition. **Antonio Arleques Gomes:** Writing – review & editing, Supervision, Data curation. **Wagner de Rossi:** Writing – review & editing, Methodology, Data curation. **Jing Wang:** Resources, Funding acquisition, Formal analysis. **Arian Pérez Nario:** Writing – review & editing, Formal analysis. **Qinggang He:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

Funding

This study was supported by the following grants, including the National Natural Science Foundation of China Major Research Instrument Development Project (Grant No. 32027802), the Zhejiang University Instrument Development Project (Project No. YQZX-B202407), Zhejiang Provincial "Lingyan" Research and Development program (Grant No.2024C03100), and the CNPq (INTERAS 406761/2022-1; GD 174673/2023-0; DT 305237/2024-1).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank the radiochemistry groups of the second affiliated hospital of Zhejiang University and Jirui Technology Co., Ltd. for providing the positron emission tomography (PET) tracers.

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