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ARTICLE

A Spot Test for Determination of Residual TBA Levels in ^{18}F-Radiotracers for Human Use using Dragendorff Reagent

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When utilizing [^{18}F]tetrabutylammonium fluoride ([^{18}F]TBAF) in the synthesis of ^{18}F-labeled radiotracers for clinical positron emission tomgraphy (PET) imaging, it is necessary to confirm that residual TBA levels in formulated doses do not exceed established specifications (≤2.6 mg/patient dose). Historically this has been accomplished using HPLC, but this is time consuming for short-lived PET radiotracers and limited by the need for expensive equipment. This motivated us to introduce a TLC spot test for determining residual TBA, and we have developed a new method which employs the Dragendorff reagent. Herein we report details of the TLC method and use it to quantify residual TBA in different formulations of 6-[^{18}F]fluoro-DOPA.

Introduction

The past 10 years have seen a dramatic increase in the application of positron emission tomography (PET) imaging to both enhancing medical care and accelerating drug discovery. Reflecting this, about two million positron emission tomography (PET) scans are performed annually in the US. PET scans utilize bioactive molecules tagged with a radionuclide (radiotracers),[1] and fluorine-18 is frequently the PET radionuclide of choice owing to: (i) the attractive imaging properties of ^{18}F (97% β^+ decay), (ii) the ready availability of ^{18}F from small medical cyclotrons, (iii) the 110 min half-life of ^{18}F, which enables commercial distribution to satellite imaging centers, and (iv) the prevalence of fluorine in drug molecules.[2,3] Historically, PET imaging has been dominated by [^{18}F]fluorodeoxyglucose ([^{18}F]FDG) following its approval by the US Food and Drug Administration (FDA) and reimbursement by the Centers for Medicare and Medicaid (CMS) in the 1990s. Many of the developments in synthesis, quality control (QC) testing and regulatory oversight pertaining to fluorine-18 radiochemistry have thus been steered by the need to manufacture [^{18}F]FDG for widespread clinical use according to current Good Manufacturing Practice (cGMP). For example, synthesis of [^{18}F]FDG (and many historical radiotracers) involves use of kryptofix-2.2.2 ($K_{2.2.2}$) as a phase transfer catalyst to enhance the reactivity of nucleophilic [^{18}F]KF, and over ten years ago we reported a thin layer chromatography (TLC) method for analyzing residual $K_{2.2.2}$ levels in formulated radiotracer doses.[4] However, increases in utilization of PET are, in part, being driven by demand for new radiotracers. The last 10 years has seen FDA approval of several new ^{18}F-labeled radiotracers for PET imaging of amyloid plagues (Amyvid, Vizamyl, Neuraceq), tau (Tauvid), prostate cancer (auxumin), and breast cancer (Cerianna),[5] as well as an increase in the use of labeled drug assets to support pharmaceutical research and discovery.[6]

This expansion in the utilization of PET has created a need to radiolabel more diverse and complex molecules which, in turn, has spurred development of new methods for incorporating fluorine-18 into bioactive molecules.[7] For example, recent efforts have sought to improve the late-stage labeling of (hetero)arenes with high molar activity [^{18}F]fluoride. In particular, transition metal-mediated reactions using high molar activity [^{18}F]fluoride have changed the way radiochemists form C–^{18}F bonds,[8] and copper-mediated radiofluorination (CMRF) has proven one of the most versatile of such approaches to date (for a review of radiotracers synthesized by CMRF, see:[9]). Key to the development and optimization of new radiofluorination reactions in our laboratory has been venturing beyond the traditional [^{18}F]KF•$K_{2.2.2}$ paradigm to explore new elution strategies[10] and alternate sources of [^{18}F]fluoride such as [^{18}F]AgF, [^{18}F]HF and [^{18}F]tetrabutylammonium fluoride ([^{18}F]TBAF).[11–14]

In our recently reported one-pot nucleophilic synthesis of 6-[^{18}F]fluoro-DOPA ([^{18}F]FDOPA) by CMRF, [^{18}F]TBAF was utilized as the [^{18}F]fluoride source.[13,14] Use of [^{18}F]TBAF for such clinical radiotracer production presents different quality control considerations to using [^{18}F]KF•$K_{2.2.2}$. There is no limit for residual TBA salts in radiotracer doses defined by the FDA, and so typically U.S. radiotracer manufacturing facilities adopt the limit of 2.6 mg/patient dose (*V*) set by the European Pharmacopeia (Ph. Eur.). Therefore, the level of residual TBA salts in a dose needs to be determined during QC testing and must be ≤2.6 mg/*V* before doses can be released to the clinic for

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TBA (mg/ml)	Iodine	Iodoplatinate	Manually prepared **Dragendorff**
10	Rust orange spot	Solid redwood spot	Solid orange spot
1	faint orange spot	Solid redwood spot with gray halo	Faint orange spot
0.5	faint orange halo	Solid redwood spot with gray halo	Orange halo
0.26 (standard 10 mL dose limit)			
0.25	faint orange halo	Solid redwood spot with gray halo	Orange halo
0.1 (26 mL dose limit)	ND	ND	Faint orange halo
0.9 (28 mL dose limit)			
0.01	ND	ND	Faint orange halo
0.001	ND	ND	ND

Table 1: Various TLC Stains for the detection of TBA (ND = not detectable)

administration to a patient. Historically, residual TBA levels have been difficult to determine, with the Ph. Eur. suggesting use of high performance liquid chromatography (HPLC) to perform the analysis. While a number of HPLC methods have been reported,[15,16] the approach is expensive especially since it likely requires a dedicated system in a cGMP environment, time consuming (not ideal when working with short-lived radionuclides), and therefore not without its limitations.[17] As such, radiochemists have been motivated to develop a TLC test akin to that used to determine residual $K_{2.2.2}$ levels.[4,18–20] While it is possible to conduct quantitative TLC analysis by spotting multiple concentrations of standards,[21] when conducting QC testing of short-lived radiotracer doses we prefer simple go/no TLC spot-tests. Spot-tests allow rapid confirmation that impurity levels do not exceed established limits,[4] although we note that care should be taken to ensure accurate spotting when only using a single spot to test a dose.

Kuntzsch and colleagues developed a method in which plates were treated with MeOH/NH_4OH and stained with iodine.[19] However, the reported TBA limit of detection (LoD) of 0.04 mg/mL is well below the dose limit, making it difficult to assess a clear pass or fail result. In addition, plates need to be treated with MeOH/NH_4OH prior to iodine staining and routine use of iodine vapors requires a containment hood for safe use at our institution. More recently, Halvorsen and Kvernenes adapted the classical iodoplatine reagent used in $K_{2.2.2}$ analysis for detecting TBA.[22] Although this method is useful for testing standard 10 mL doses (V = 10, assuming the entire dose is administered to a single patient), corresponding to a concentration of TBA at 0.26 mg/mL, the method's LoD cannot be altered. It is therefore challenging to use for other injection volumes (V), which have different TBA concentrations. Notably, in addition to our new CMRF method for preparing [^{18}F]FDOPA in 10 mL saline,[13,14] certain existing clinical trials require that we synthesize [^{18}F]FDOPA in 28 mL of phosphate-buffered saline (PBS) using the traditional S_NAr synthesis approved by FDA for the trial (corresponding to a TBA concentration of 0.09 mg/mL).[23] This is a more dilute formulation than is perhaps typical for radiotracers, but such formulations are becoming increasingly common for radiotracers prepared using the newest generation of cassette-based synthesis modules.[22,23] The iodoplatinate test could be adequate with a stipulation limiting the injection volume to 10 mL of the 28 mL dose (i.e., V = 10 mL), but could not be used if a need exists to administer the entire dose (V = 28 mL) because of, for example, distribution to a distant imaging site. As such, we were in need of a TLC method to determine residual TBA levels in different formulations and volumes of [^{18}F]FDOPA, and have developed a fast and quantitative spot test that employs the classical Dragendorff stain.[24,25] This new method has high specificity for TBA (compared to the radiotracer and other formulation components) and, because it can be customized for different formulation volumes, it is applicable to a wide range of radiotracer doses including both formulations of [^{18}F]FDOPA used for clinical imaging at our institution. The TLC spot test requires minimal equipment and can be completed quickly within the constraints of PET radiotracer quality control, which usually needs to be completed ≤20 min. At TBA levels ≥2.6 mg/V the spot test results in an easily detectable spot, while at concentrations ≤2.6 mg/V it does not, allowing for easy go/no-go decisions on dose release to be made during QC testing.

2. Experimental

2.1 Materials and Methods

All reagents were of the highest purity or pharmaceutical grade and used as received, without further purification. Tetrabutylammonium trifluoromethanesulfonate (≥99.0%, part no. 86888), potassium iodide (for analysis EMSURE® ISO, Reag. Ph Eur, part no. 1.05043), bismuth (III) nitrate pentahydrate (98%, part no. 248592), commercial Dragendorff reagent and spray solution (for TLC, part nos. 44578 and 1.02035) and tetraethylammonium (TEA) bicarbonate (≥95.0%, part no. 11268) were purchased from Millipore-Sigma; Nitric Acid (TraceMetal grade, part no. A509), 12 N sodium hydroxide (certified & NIST traceable, part no. LC245232) and 6 N hydrochloric acid (certified & NIST traceable, part no. SA56) were purchased from Fisher Scientific; $K_{2.2.2}$ (98%, part no. 291950010) was purchased from Acros; 0.9% Sterile saline, (USP grade, NDC: 0409-4888-03) was purchased from Hospira. PBS was provided with the ABX [^{18}F]FDOPA FASTLab kit. Watman filter papers (5.5 cm diameter, part no. 1001055) were purchased from Millipore-Sigma. Standards were prepared by dissolving and diluting TBAOTf in either water, saline, or PBS. $TEAHCO_3$ was dissolved in distilled water. TLC was carried out on glass-backed silica gel plates (Millipore part no. 1.05715.0001; 3.0 x 7.5 cm; F_{254} Silica Gel 60 Å; layer thickness 250mm). TLC plates were spotted with 2 µL samples using an auto-pipette (Rainin 0.5–10ml). TLC plates were stained using either iodine vapor, iodoplatinate reagent, commercially available Dragendorff solution, or manually prepared Dragendorff solution.

2.2 Preparation of Dragendorff solution

Stock Dragendorff solution was prepared according to a literature procedure[26]:

Solution A:

(a) 8.0 g bismuth(III) nitrate was dissolved in 25 mL 25% Nitric Acid (bismuth solution);
(b) 20 g potassium iodide was used to make a slurry in 1 mL 6 N HCl and 5 mL water (i.e. KI does not totally dissolve);
(c) The bismuth solution was added to the slurry slowly while maintaining stirring;
(d) The resulting solution was diluted with 100 mL water and any solid present was removed by filtration (Watman 5.5 cm filter papers).

Dragendorff Stock Solution:

In a solution containing 20 mL water and 5 mL 6 N HCl was added 2 mL of ***Solution A***, followed by 6 mL 6N NaOH and the resulting mixture was shaken. In the event that residual bismuth hydroxide solid could not be dissolved, additional 6 N HCl (typically 5 – 20 drops) were added until a yellow-orange translucent solution was obtained.

Diluted Dragendorff Solution:

Dragendorff Stock Solution was diluted 1:15 or 1:9 in H_2O and a cloudy solution was formed. 6 N HCl was then added dropwise to the diluted stain solution until a transparent yellow solution was formed.

2.3 TLC Procedure

2 µL spots of TBA standards or formulated dose were applied to silica plates or pre-developed plates containing iodoplatinate solution via an auto-pipette. In the case of Dragendorff or iodine staining, the spots were then dried with a cool air stream for 30 seconds. For Dragendorff staining, the TLC plates were dipped into the Dragendorff solution to fully immerse the spots for 10-20 seconds to allow for the formation of orange precipitate. Once removed, plates were photographed immediately and visually analyzed. Air drying after the Dragendorff staining can enhance the intensity of the spots, however using warm air resulted in a whiting out of the plate.

2.4 Preparation of TLC Standards

A 1 mg/mL TBA solution was generated by dissolving 16.15 mg of TBAOTf in 10 mL of either distilled water, saline, or PBS. A series of 1 mL TBA standard concentrations were prepared by serial dilution of the 1 mg/mL solution in its appropriate buffer to generate a range of concentrations from 0.3 mg/mL – 0.05 mg/mL. A 1 mg/mL TEA solution was generated by dissolving 14.7 mg of $TEAHCO_3$ in 10 mL of distilled water. A 0.05 mg/mL $K_{2.2.2}$ solution was generated by dissolving 2.5 mg of $K_{2.2.2}$ in 50 mL of distilled water.

2.5 Synthesis of [^{18}F]FDOPA with TRACERlab $FX_{N\text{-}Pro}$.

[^{18}F]FDOPA was prepared as previously described.[13,14,23]

3. Results and discussion

In our recently developed synthesis of [^{18}F]FODPA using CMRF, 7.5 mg of TBAOTf is used to generate [^{18}F]TBAF,[13,14] while the commercially cassettes available for production of [^{18}F]FODPA utilize 24 mg $TBAHCO_3$.[23] As such, in the event of a purification problem it is possible that levels of TBA in the final product prepared using either method exceed the established concentration limit defined by *Ph Eur* (2.6 mg/*V*, where *V* = 10 mL[13,14] or 28 mL,[23] respectively). It should be noted that for the different formulation volumes the limit, and thus the sensitivity of the test, will vary (e.g. limits in 28 and 10 mL doses are of 0.09 and 0.26 mg/mL, respectively, assuming the entire dose is administered to a single patient).

In our initial studies, we considered known TLC stains for quaternaryalkyl ammonium cations (e.g. TBA). Three of the most promising, iodoplatinate that is used for $K_{2.2.2}$, Dragendorff stain (potassium bismuth iodide), and iodine vapor were tested for their ability to visualize TBA at different concentrations between 0.001 and 10 mg/mL (**Table 1** and **Fig. 1**). Standards were prepared by serial dilution of TBAOTf in water, and the LoD for each TLC stain was determined.

Visualization of TBA using pre-developed iodoplatinate plates proved challenging in our hands and results obtained were difficult to interpret (**Fig. 1a**). The staining pattern of TBA was difficult to distinguish from a control spot (water) with the same colored concentric circles surrounding the spot of interest observed. Iodine staining showed a robust spot at the 10 mg/mL TBA concentration, but quickly lost intensity with further dilution (**Fig. 1c**). Although iodine and iodoplatinate staining of TBA indicated positive staining at ≥0.25 mg/mL TBA, they did not provide a positive stain at 0.1 mg/mL, meaning they are not suitable tests for larger more dilute formulations that are common in multidose preparation of fluorine-18 radiotracers.[23] Iodine staining has been published as a viable method for TBA assessment, but requires the addition of 10 µL of $MeOH/NH_4OH$ (90:10 v/v) to the TBA spot in order to enhance the signal and improve the LoD.[19] Our goal was to establish a quick TLC method that did not require more than just spotting the solution of interest and applying a stain in order to reduce complexity and minimize potential for test error. We therefore shifted our focus to investigating the Dragendorff stain for analysis of TBA.

Dragendorff reagent is commercially available as a spray solution or as an even more concentrated dipping solution (see *Materials and Methods, Section 2.1*), and is known to have specificity for alkaloids and quaternaryalkyl ammonium bases.[27] The specificity of Dragendorff reagent for TBA proceeds through a single displacement reaction, with TBA thought to exchange with potassium in the active ingredient ($KBiI_4$) to generate an easily visualized orange precipitate (eq. *1*).[28]

$$[Bu_4NH]^{18}F + K[BiI_4] \longrightarrow [^{18}F]KF + [Bu_4NH][BiI_4] \quad (1)$$

Insoluble Orange Salt

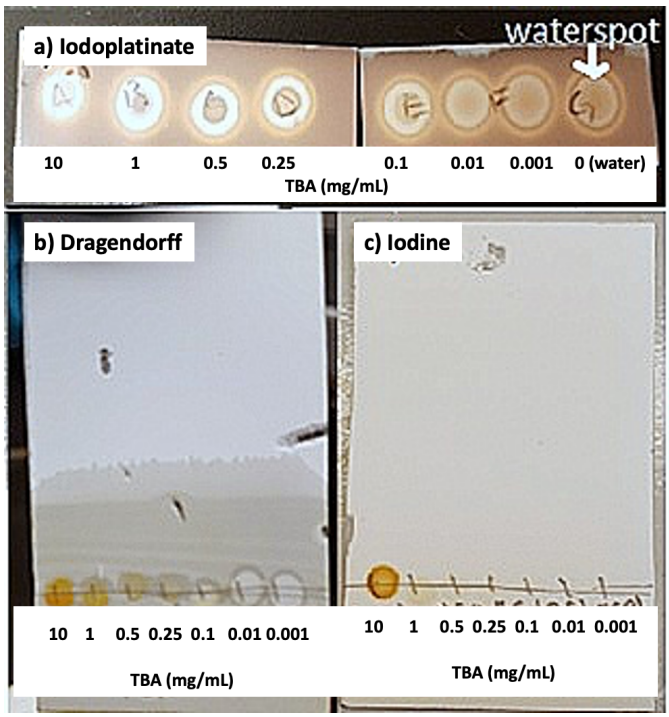

Fig. 1 TLC Staining of TBA standards using a) pre-developed iodoplatinate plates, b) manual Dragendorff stain, and c) iodine chamber

Use of either commercially available Dragendorff spray or dipping solution resulted in a pale orange background on which to interpret a positive stain, with an LoD of 0.14 mg/mL TBA (**Fig. 2**). We concluded that the orange background would make it difficult to accurately and rapidly quantify the presence of residual TBA in radiotracer doses, particularly at low concentrations, and efforts to customize these commercially available solutions for our needs have thus far been unsuccessful. The preparation of a custom Dragendorff stock solution was therefore undertaken in order to tune its sensitivity and eliminate the background color for higher spot contrast. After optimization, our prepared Dragendorff reagent (see *Experimental* section for details), provided positive orange spots with a white background and an LoD of 0.01 mg/mL (**Fig. 1b**). The TLC stain provided a clear background for confident identification of a positive spot at (or above) the allowable limit for injection (LoI). A semiquantitative TLC method for TBA using prepared Dragendorff solution was further developed. Although the active ingredient (BiI_4^-) remains the same in both the commercial products and our custom solution, the use of acetic acid and ethyl acetate as solvents in the commercial products (versus nitric acid and water used to prepare our version) may contribute to the orange background seen with the commercial stains (**Fig. 2**). The exact contents of commercially available Dragendorff reagent are proprietary, limiting further speculation.

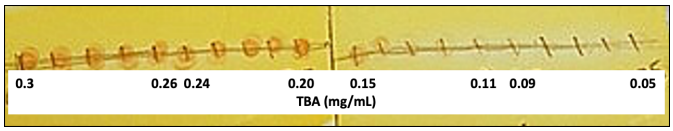

Fig. 2 Representative commercial Dragendorff stain of TBA standards

In order to develop a quick pass or fail spot test for TBA in different radiotracer formulations, we hypothesized that the LoD of Dragendorff stain could be varied by dilution to match the appropriate LoI for TBA in a given formulation volume. A test where any TBA concentration above the LoI would give a positive response and any concentration below would not yield a spot by staining was the goal of our development. As proof of concept, we wished to develop tests for analyzing residual TBA levels in the two formulations of [^{18}F]FDOPA utilized in our laboratory (10 mL of saline[13,14] and 28 mL of PBS[23]). We prepared standards spanning the TBA LoD (≤ 0.26 mg/mL) for a 10 mL dose formulated in normal saline (**Table 2** and **Fig. 3a**, A-K) as well as standards spanning the LoD (≤0.1 mg/mL) for a 28 mL dose formulated in PBS (**Table 2** and **Fig. 3b**, L-V), and explored development of custom Dragendorff stains for both.

Diluting our custom Dragendorff solution with water (1:15 when staining TBA standards representative of [^{18}F]FDOPA formulated in 10 mL saline, and 1:9 when staining standards representative of FDOPA dissolved in 28 mL of PBS) proved optimal and solid orange spots were observed down to the LoI for both formulations (0.23 mg/mL (**Fig. 3a**) and 0.08 mg/mL (**Fig. 3b**), respectively). Gratifyingly, no matrix interference was observed from saline or PBS. This demonstrated the robustness of the prepared Dragendorff reagent spot test and the ability to customize it for a given radiotracer formulation. Of note, neither [^{18}F]FDOPA formulation contained organic components such as ethanol, which is commonly used to formulate radiotracers.[29] Future work for will evaluate whether organic solvents interfere with the test.

Table 2: TBA concentration identifiers A-V

Identifier	A	B	C	D	E	F	G	H	I	J	K
TBA (mg/mL)	0.3	0.29	0.28	0.27	0.26	0.25	0.24	0.23	0.22	0.21	0.2
Identifier	L	M	N	O	P	Q	R	S	T	U	V
TBA (mg/mL)	0.15	0.14	0.13	0.12	0.11	0.1	0.09	0.08	0.07	0.06	0.05

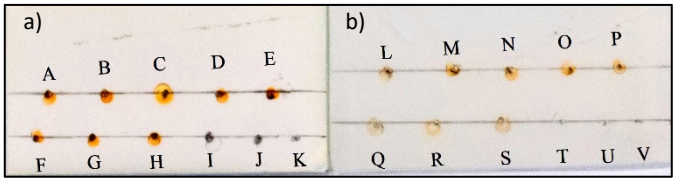

Fig. 3 Dragendorff stain of a) saline prepared TBA standards and b) PBS prepared TBA standards

With a pass or fail TLC spot test for TBA in hand, we further investigated the utility of the method. First, we checked for applicability beyond TBA, and were gratified to observe that the method also appears suitable for testing of residual levels of TEA salts[30] and $K_{2.2.2}$ (**Fig. 4**); future work will establish optimal dilutions and LODs for both. Next, we used the stain to analyze residual TBA levels in [^{18}F]FDOPA batches prepared for clinical use using either a GE TRACERlab FX_{FN} [13,14] or a GE FASTlab2[23] and formulated in 10 mL saline (n = 3) or 28 mL of PBS (n = 4), respectively (**Table 3**). To test for the possibility of false negative results, an aliquot of the final dose (1 mL) was directly spiked with an internal TBA standard corresponding to the LoI (0.09 or 0.26 mg). By spotting the final dose, the final dose containing an internal TBA standard, a TBA standard equal to that of the LoI and a negative control (water) on the same plate, it was possible to determine with confidence that doses of

[18F]FDOPA prepared via either method did not contain TBA above the LoI, and the doses were thus suitable for human use (**Fig. 5**).

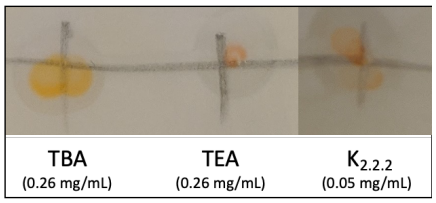

Fig. 4 Analysis for other potential contaminants in radiotracer formulations using Diluted Dragendorff Stain (1:9)

Table 3: QC Analysis of [18F]FDOPA

[18F]FDOPA Formulation	LoI (mg/mL)	Dose	Standard	Dose spiked with Std	Water	n
10 mL Saline	≤0.26	○	●	●	○	3
10 mL Saline	≤0.26	○	●	●	○	1[a]
28 mL PBS	≤0.09	○	●	●	○	4

[a] using 14 d old Dragendorff reagent

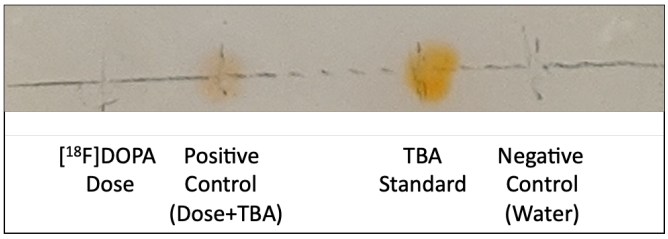

Fig. 5 Representative Dragendorff testing of a 10 mL [18F]FDOPA dose

Finally, the longevity of the stain was tested by performing analysis of TBA standards (dissolved in both saline and PBS) every other day for two weeks using the *Diluted Dragendorff Solution* (see Section 2.2). The stain was kept in a fume hood at room temperature during the 2-week test period. The same results were obtained over the 2-week duration (**Table 3**), indicating that our custom Dragendorff stain is shelf stable and can be used for routine radiotracer QC testing. Decomposition of both the diluted and stock solutions occurred after a month (notably when exposed to light or air), and so we recommend a 1-month expiration time.

4. Conclusions

In summary, a quick reliable TLC spot test for determining residual TBA levels in radiotracer formulations has been developed using the Dragendorff reagent. The test is straightforward, does not require expensive equipment to implement, can easily be tuned for different radiotracer formulations, and is analogous to existing TLC spot tests for $K_{2.2.2}$ allowing easy introduction at PET Centers using [18F]TBAF to produce clinical radiotracers. This spot test is also expected to facilitate use of [18F]TBAF at more facilities in the future as it allows easy QC testing without causing work flow issues or mandating costly equipment acquisitions. Our facility has implemented this TLC spot test for analysis of residual TBA in [18F]FDOPA prepared for clinical use with [18F]TBAF. Future efforts will focus upon formal method validation for routine use of the TLC stain in a cGMP setting (to determine, for example, sensitivity, accuracy, precision, and robustness),[31,32] as well as digitalization approaches for capturing TLC data to report in synthesis master batch records.[33]

Conflicts of interest

The authors declare no conflicts of interest.

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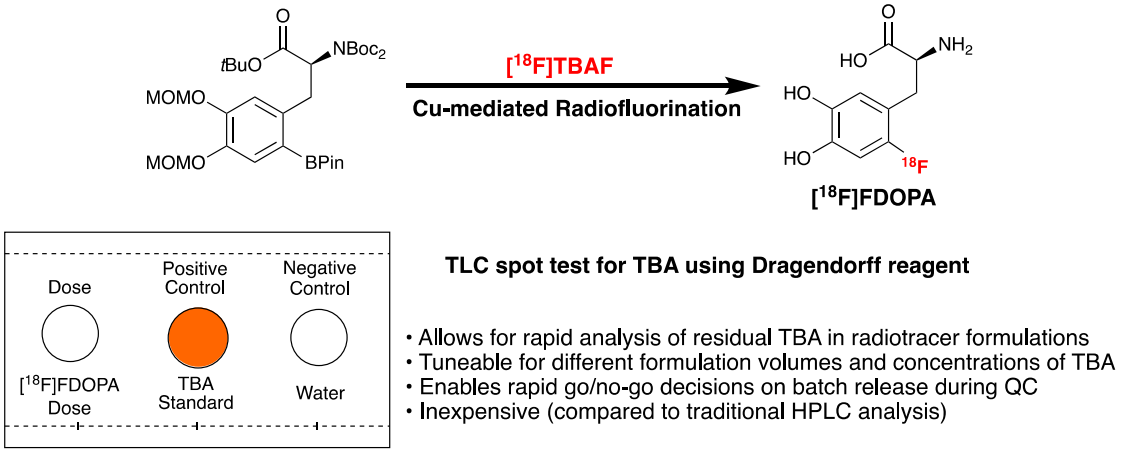

A TLC spot test for determining residual TBA levels in positron emission tomography (PET) radiotracer formulations has been developed. The new method employs the Dragendorff reagent, and proof-of-concept is demonstrated through quantification of residual TBA amounts in different formulations of 6-[^{18}F]fluoro-DOPA.