Validation of analytical HPLC with post-column injection as a method for rapid and precise quantification of radiochemical yields.

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Abstract:

Accurate assessment of isolated radiochemical yields (RCYs) is a prerequisite for efficient and reliable optimization of labeling reactions. In practice, radiochemical conversions (RCCs) determined by HPLC analysis of crude reaction mixtures are often used to estimate RCYs. However, incomplete recovery of radioactivity from the stationary phase can lead to significant inaccuracies if RCCs are calculated based on the activity eluted from the column (i.e. the summed integrals of all peaks). Here, we validate a simple and practical method that overcomes problems associated with retention of activity on the column by determination of the total activity in the sample using post-column injection. Post-column injections were carried out using an additional injection valve, which was placed between the outlet of the HPLC column and the inlet of the detectors. 2-[^{18}F]Fluoropyridine ([^{18}F]FPy) and 8-cyclopentyl-3-(3-[^{18}F]fluoropropyl)-1-propylxanthine ([^{18}F]CPFPX) were prepared with radiochemical purities of >99.8% and mixed with [^{18}F]fluoride at a ratio of 1:1 to simulate reaction mixtures obtained by radiolabeling reactions with an RCC of 50%. The samples were analyzed on three different C_{18} HPLC columns using neutral and acidic mobile phases. RCCs determined using the summed area of all peaks in the chromatograms were compared with those determined using post-column injection. Additionally, RCCs determined by post-column injection were corrected for activity losses before, during and after radiosyntheses to afford analytical RCYs, which were compared with isolated RCYs. Determination of RCCs based on the summed area of all peaks gave correct results under certain chromatographic conditions, but led to overestimation of the actual RCCs by up to 50% in other cases. In contrast, determination of RCCs using post-column injection provided precise results in all cases, and often significantly reduced analysis time. Moreover, analytical RCYs calculated from RCCs determined by post-column injection showed excellent agreement with isolated RCYs (<3% deviation). In conclusion, HPLC analysis using post-column injection enables reliable determination of RCCs independent of the chromatographic conditions and, together with a simple activity balance, rapid and accurate prediction of isolated RCYs.

Keywords:

radiochemical conversion (RCC), high performance liquid chromatography (HPLC), positron emission tomography (PET) tracer, fluorine-18, radiolabeling efficiency, radiochemical yield (RCY)

1. Introduction

A broader implementation of positron emission tomography (PET) into clinical diagnostics critically depends on the accessibility of established and emerging PET-tracers. As a consequence, optimization of existing and development of novel radiolabeling procedures represent important aspects of applied research in radiochemistry. Both rely heavily on fast and accurate methods to assess labeling efficiencies due to the short half-lives of typical PET-nuclides (e.g. ^{11}C, ^{18}F or ^{68}Ga) and the tiny amounts of radionuclides used in no-carrier-added (n.c.a.) radiosyntheses. While the product activity obtained from a given starting activity of radionuclide without decay-correction (i.e., activity yield) is the most important criterion for the efficacy of radiotracer production, radiochemical yields (RCYs), which are corrected for decay, are typically used as efficiency measures in basic research. With regard to the overall process efficiency of radiosynthetic procedures, the isolated RCY determined after purification of the product is typically considered as the gold standard [1]. However, the development of novel protocols for PET-tracer production often involves extensive optimization studies that can encompass dozens or even hundreds of radiolabeling experiments. In this case, determination of the labeling efficiency by chromatographic analysis of crude reaction mixtures is often used to circumvent laborious and time-consuming isolation of the labeled products after each optimization experiment. Accordingly, radiochemical conversions (RCCs), which refer to the content of the product in the reaction mixture before isolation, are widely applied as a convenient benchmark for the reaction efficiency of a specific radiolabeling step [2].

The most popular method to quantify radioactive products by high performance liquid chromatography (HPLC) is based on the assumption that the sum of all peaks in a chromatogram (s.o.p.) amounts to the total activity in the analyzed sample. However, this assumption may not be valid in the case of radiofluorination reactions with [^{18}F]fluoride, since [^{18}F]F^- often shows undesirable chromatographic properties in reversed phase HPLC (RP-HPLC), such as incomplete recovery from the column and poor peak shapes [3]. Similar effects can occur with radiometals like ^{68}Ga [4]. Besides, other processes associated with retention of radioactive materials on the stationary phase like the formation of precipitates and/or highly lipophilic by-products can contribute to incomplete recovery of radioactivity from the column. If not accounted for, all of these effects can result in an underestimation of the total activity in a sample and, consequently, an overestimation of the RCC or the radiochemical purity (RCP) of the product.

In order to overcome these limitations and precisely determine the RCC or RCP by a single HPLC measurement, we have used post-column injection (p.c.i.) to quantify the total activity in a sample. For this method, the HPLC system is equipped with a second injection valve placed

Abbreviations: [^{18}F]CPFPX, 8-cyclopentyl-3-(3-[^{18}F]fluoropropyl)-1-propylxanthine; [^{18}F]FPy, 2-[^{18}F]fluoropyridine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; Et_4NOTf, tetraethylammonium trifluoromethanesulfonate; H_2O, water; HPLC, high performance liquid chromatography; K_2CO_3, potassium carbonate; MeCN, acetonitrile; MeOH, methanol; NaOH, sodium hydroxide; n.c.a., no-carrier-added; PET, positron emission tomography; RCC, radiochemical conversion; p.c.i., post-column injection; PTFE, polytetrafluoroethylene; RCP, radiochemical purity; RCY, radiochemical yield; RP-HPLC, reversed phase HPLC; s.o.p., sum of peaks; SPE, solid-phase extraction; TFA, trifluoroacetic acid; TLC, thin layer chromatography.

between the outlet of the HPLC column and the inlet of the detectors as illustrated in the graphical abstract. An additional sample aliquot injected through this valve bypasses the stationary phase, so that the resulting single peak can be utilized to accurately determine the total activity in the sample. Although this method is routinely used by us and others (for recent examples see: [5,6]), its comprehensive validation is still lacking. Herein, it is demonstrated that radio-HPLC with post-column injection can be applied to accurately quantify the RCC or RCP regardless of adsorption processes on the stationary phase. In addition, RCCs determined by radio-HPLC with post-column injection, in conjunction with an activity balance to account for losses of radioactivity before, during and after the radiolabeling reaction, can be used for accurate prediction of isolated RCYs.

2. Material and Methods

2.1. General

Analytical HPLC was performed on a HPLC system (Knauer, Berlin, Germany) consisting of an Azura P 6.1L pump, Rheodyne 7725i injection valves equipped with original 20 µL steel loops, and an Azura UVD 2.1S UV detector coupled in series with a HERM LB 500 2'' NaI radiation detector with digital signal transmission (Berthold Technologies, Bad Wildbad, Germany). Data acquisition was performed with Knauer ClarityChrom 8 software.

The following analytical HPLC columns were used: Chromolith® SpeedROD RP18e 4.6×50 mm (Merck KGaA, Darmstadt, Germany), MultoKrom® 100-5 C18 4.6×250 mm (CS-Chromatographie Service GmbH, Langerwehe, Germany), and SunFire® C18 5 µm, 4.6×150 mm (Waters GmbH, Eschborn, Germany).

Semi-preparative HPLC was performed on a HPLC system consisting of a 40P Pump (Knauer, Berlin, Germany), a Rheodyne 7725i injection valve equipped with a 2 mL steel loop, and an Azura UVD 2.1S UV detector coupled in series with a custom-made Geiger counter. Data acquisition was performed with the custom JuHPLC software.

Thin layer chromatography (TLC) was performed on aluminum-backed Si-60 plates with fluorescence indicator. Radio-TLC scans were visualized by a PerkinElmer Cyclone® Plus phosphor imaging system and the OptiQuant 5.0 software. After development of the TLC plates, they were air-dried for 2–3 min and then covered with plastic foil before exposing them to the film.

All reagents and HPLC gradient grade solvents were purchased from Merck KGaA (Darmstadt, Germany) or Sigma Aldrich (Taufkirchen, Germany). Type 1 ultrapure water (H_2O) with a resistivity > 17 MΩ*cm^{-1} obtained from an Elga Purelab Classic system (ELGA LabWater Veolia Water Technologies Deutschland GmbH, Celle, Germany) was used in all experiments.

2.2. Radiochemistry

[^{18}F]Fluoride was produced via the ^{18}O(p,n)^{18}F nuclear reaction by bombardment of 98% ^{18}O-enriched [^{18}O]H_2O (Rotem Industries Ltd., Arava, Israel) with 16 MeV protons in a 1.6 mL silver

liquid target using a GE PETtrace™ 800 cyclotron (GE Healthcare GmbH, Munich, Germany). Radioactivity was measured with a Curiementor 2 from PTW GmbH (Freiburg, Germany).

Labeling reactions were performed in 5 mL V-Vials equipped with a polytetrafluoroethylene (PTFE)-coated stirring bar and a silicone septum. Solutions were handled with disposable medical syringes and cannulas. All temperatures refer to the temperature of the metal heating block. Sep-Pak Accell Plus QMA carbonate plus light cartridges (130 mg sorbent; Waters GmbH, Eschborn, Germany) were used as obtained. The irradiated [^{18}O]H_2O was diluted with ultrapure H_2O to a volume of 1 mL prior to [^{18}F]fluoride processing.

2.2.1. Radiosynthesis of 8-cyclopentyl-3-(3-[^{18}F]fluoropropyl)-1-propylxanthine ([^{18}F]CPFPX)

[^{18}F]CPFPX was prepared from the corresponding pivaloyloxymethyl (POM)-protected tosylate precursor as reported elsewhere [7] with minor modifications as follows. [^{18}F]Fluoride was loaded onto a QMA cartridge from the male side and eluted from the female side using a mixture of K2.2.2 (9 mg) and 1 M potassium carbonate (K_2CO_3) (12 µL) in 70% acetonitrile (MeCN) (1 mL). After evaporation of the solvents at 85 °C in a stream of argon, azeotropic drying was performed twice by adding anhydrous MeCN (1 mL) followed by evaporation for 3 min at < 20 mbar. A solution of the labeling precursor (4 mg) in anhydrous dimethyl sulfoxide (DMSO) (500 µL) was then added, the mixture was stirred at 85 °C for 10 min, and the reaction vial was cooled in a water bath at ambient temperature for 1 min to < 40 °C. 2 M sodium hydroxide (NaOH) (200 µL) was added and stirring was continued for 3 min. Thereafter, the reaction mixture was diluted with H_2O (1 mL) and trifluoroacetic acid (TFA) (60 µL). An aliquot of the reaction mixture (5–10 µL) was diluted with HPLC mobile phase (for composition see section 2.3 and Tab. 1) and used for determination of the RCC. The remaining reaction mixture was simultaneously subjected to semi-preparative HPLC [column: Hydro RP 10 µm 10×250 mm (Phenomenex Ltd, Aschaffenburg, Germany), gradient: 0–12 min: H_2O, 12–30 min: 40% MeCN, flow rate: 7.4 mL/min, t_R: 23–25 min] to afford purified [^{18}F]CPFPX. Radio-TLC of [^{18}F]CPFPX was performed using 40% ethyl acetate in cyclohexane as the solvent.

2.2.2. Radiosynthesis of 2-[^{18}F]fluoropyridine ([^{18}F]FPy)

[^{18}F]FPy was prepared from commercially available 2-nitropyridine using “minimalist” processing of the [^{18}F]fluoride with tetraethylammonium trifluoromethanesulfonate (Et_4NOTf) [8]. Accordingly, [^{18}F]fluoride was loaded onto a QMA cartridge from the male side, the cartridge was flushed in the same direction with anhydrous methanol (MeOH) (2–3 mL) and eluted from the female side with a solution of Et_4NOTf (2 mg, 5.1 µmol) in MeOH (500 µL). MeOH was evaporated within 3–5 min under reduced pressure (300 mbar) in a stream of argon at 110 °C, and a solution of the precursor (2 mg) in anhydrous dimethylformamide (DMF) (500 µL) was added. The mixture was stirred at 110 °C for 10 min, and the reaction vial was cooled for 2 min in an ice bath to < 40 °C before H_2O (1 mL) was added. An aliquot of the reaction mixture (5–10 µL) was diluted with HPLC mobile phase (for composition see section 2.3 and Tab. 1) and used for determination of the RCC. The remaining reaction mixture was simultaneously subjected to semi-preparative HPLC [column: Hydro RP 10 µm 10×250 mm (Phenomenex Ltd, Aschaffenburg, Germany), gradient: 0–6 min: H_2O, 6–30 min: 18% MeCN, flow rate 7.4 mL/min, t_R: 15–16 min] to afford purified [^{18}F]FPy. Radio-TLC of [^{18}F]FPy was performed using 5% ethyl acetate in cyclohexane as the solvent.

2.2.3. Preparation of test samples with known RCP/RCC

For preparation of the test samples, equal volumes of the respective tracer solution obtained by semi-preparative HPLC purification and [^{18}F]fluoride in [^{18}O]H_2O were dispensed into identical vials (to avoid errors due to self-absorption and geometrical effects during activity measurements). Volumes of the solutions corresponding to equal activities (±0.2%; determined using a dose calibrator) were then combined to give the model reaction mixtures.

2.3. Radio-HPLC analysis

[^{18}F]FPy was analyzed using the following conditions: Chromolith® SpeedROD, gradient: 0–3 min: 2% B, 3–20 min: 8% B, flow rate: 2 mL/min; MultoKrom® and Sunfire®, gradient: 0–5 min: 5% B, 5–20 min: 20% B, flow rate: 1.5 mL/min. [^{18}F]CPFPX was analyzed using the following conditions: Chromolith® SpeedROD, gradient: 0–3 min: 2% B, 3–20 min: 33% B, flow rate: 2 mL/min; MultoKrom® and Sunfire®, gradient: 0–5 min: 5% B, 5–20 min: 48% B, flow rate: 1.5 mL/min. A: H_2O or H_2O (0.1% TFA) and B: MeCN or MeCN (0.1% TFA), respectively. For each sample injection, the sample loops (20 µL) were overfilled with at least three volumes (60 µL) and care was taken to inject no air bubbles.

Sample analyses and post-column injections were performed using the conventional sample injection loop (= pre-column injections) and a second injection loop placed between the outlet of the HPLC column and the inlet of the detectors (= post-column injections) respectively. Both injection loops were loaded separately and the post-column injections were performed at different times of the analysis depending on column length. For short columns, such as the Chromolith® SpeedROD (4.6×50 mm; Merck KGaA, Germany), the post-column injection was carried out at the end of the HPLC analysis, so that the last peak in the chromatogram corresponds to the total activity in the sample. For longer columns, the post-column injection was carried out immediately after the pre-column injection, so that the first peak in the chromatogram (eluted within the void time) corresponds to the total activity in the sample. Chromatograms were decay-corrected to the time of injection, so that the positions of the peaks and exact timing of the post-column injections did not affect the analyses. The analytical RCY was calculated according to:

$$RCY_{anal.} = RCC \times (1 - \frac{\sum A_n}{A_0}) \quad \text{[Eq. 1]}$$

where A_0 is the starting activity and A_n is the activity lost in the process step n. In the scope of this study, $\sum A_n$ was defined as the sum of:

A_i: remaining activity on the QMA cartridge

$A_{ii:}$ remaining activity in the reactor

$A_{iii:}$ remaining activity in the HPLC syringe

3. Results and discussion

Precisely defined volumes of the sample aliquots used for pre- and post-column injection are essential for accurate determination of RCCs by HPLC with post-column injection. Preferentially, both volumes should be identical so that no correction factors have to be applied during the calculations. This could be easily achieved by using identical injection valves and sample loops for pre- and post-column injection. Equal volumes of the loops were validated by replacement of the column with a capillary tube and repeated injection of aliquots from the same samples via both valves. The deviation between the mean peak integrals obtained for the two sample loops was below 0.2% and thus within the range of the standard deviation and not statistically significant (Fig. 1).

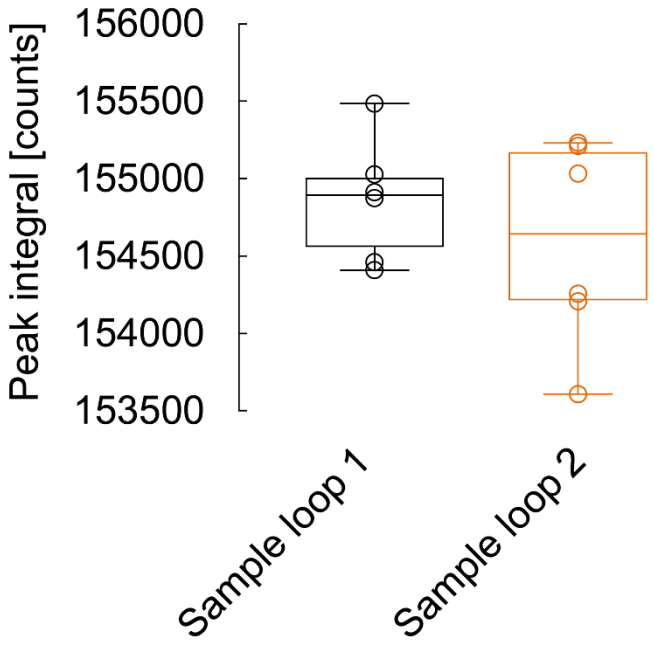

Figure 1. *Comparison of peak integrals obtained for the two injection valves. Shown are the peak integrals obtained when the HPLC column was replaced with a capillary tube and identical sample aliquots were injected via the regular injection valve (left) or the injection valve used for post-column injection (right). Boxplots indicate median, 25th and 75th percentile (box), minimum and maximum values (whiskers) and individual data points (dots). Note that there was no significant difference between the results obtained for the two injection valves, as determined by a two-tailed t-test (p=0.4).*

Another important aspect to be considered is the limited linear range of the radiation detector. Thus, due to the lack of diffusion processes on the column, a post-column injection results in a single, sharp peak with considerable peak height that could exceed the linear range of the detector at much lower sample concentrations than for the separated peaks obtained by pre-column injection. Our test system had a linear range up to 100 MBq/mL (R^2=0.9996), which is much higher than typical sample concentrations in manual radiosyntheses. However, depending on the type of detector and the signal processing technique (e.g. analog signal transmission) used, the linear range could be somewhat lower for other systems and this should be considered during sample preparation. For example, in cases where the intensity of

the post-column injection peak (as the largest peak in the chromatogram) exceeds the linear range of the detector, appropriate dilution of the sample should be used to adjust the concentration to a suitable range.

After verifying that our system meets the general requirements for radio-HPLC analyses with post-column injection, we next examined the utility of the method under various chromatographic conditions. To this end, we prepared 2-[^{18}F]fluoropyridine ([^{18}F]FPy) as a volatile and 8-cyclopentyl-3-(3-[^{18}F]fluoropropyl)-1-propylxanthine ([^{18}F]CPFPX) as a non-volatile radiolabeled model compound. Both products were purified by semi-preparative HPLC (RCP > 99.8%) and the HPLC effluents were diluted with aqueous [^{18}F]fluoride to give test samples with a defined RCP of 50±0.3%. These samples were used as surrogates for reaction mixtures obtained by radiolabeling reactions with an RCC of 50% and analyzed using different reversed phase columns and mobile phases with and without 0.1% TFA. TFA is a common additive for analysis of acidic and basic compounds, but can impede quantification of [^{18}F]fluoride by promoting formation of [^{18}F]HF (which was previously reported to be strongly retained on some reversed stationary phases [3]). For each measurement, a post-column injection was performed and the RCP was calculated based on the summed integrals of all peaks (s.o.p.) or by post-column injection (p.c.i.) respectively (Table 1). A normal phase radio-TLC analysis was also performed for comparison.

Table 1: Comparison of RCPs determined based on sum-of-peaks or post-column injection.

				RCP [%]	
Entry	**Compound**	**Stationary phase**	**Mobile phase**[a]	**s.o.p**	**p.c.i.**
1	[^{18}F]FPy	Chromolith® RP-18	H_2O / MeCN	47.4 ± 0.7	47.9 ± 1.1
2	[^{18}F]CPFPX	Chromolith® RP-18	H_2O / MeCN	45.6 ± 2.1	48.3 ± 1.5
3	[^{18}F]FPy	Chromolith® RP-18	H_2O / MeCN (0.1% TFA)	49.0 ± 1.2	48.4 ± 1.1
4	[^{18}F]CPFPX	Chromolith® RP-18	H_2O / MeCN (0.1% TFA)	48.6 ± 0.9	50.1 ± 0.4
5	[^{18}F]FPy	MultoKrom® C18	H_2O / MeCN	50.5 ± 2.0	49.1 ± 1.4
6	[^{18}F]CPFPX	MultoKrom® C18	H_2O / MeCN	49.0 ± 1.0	48.4 ± 0.6
7	[^{18}F]FPy	MultoKrom® C18	H_2O / MeCN (0.1% TFA)	55.0 ± 1.9	48.7 ± 0.6
8	[^{18}F]CPFPX	MultoKrom® C18	H_2O / MeCN (0.1% TFA)	56.9 ± 0.3	49.1 ± 0.4
9	[^{18}F]CPFPX	MultoKrom® C18	H_2O / MeCN (0.1% TFA)[b]	60.2 ± 0.4	50.0 ± 0.6
10	[^{18}F]FPy	Sunfire® C18	H_2O / MeCN	100 ± 0.0	48.6 ± 0.9
11	[^{18}F]CPFPX	Sunfire® C18	H_2O / MeCN	100 ± 0.0	59.7 ± 0.3
12	[^{18}F]FPy	Sunfire® C18	H_2O / MeCN (0.1% TFA)	100 ± 0.0	48.6 ± 0.9
13	[^{18}F]CPFPX	Sunfire® C18	H_2O / MeCN (0.1% TFA)	100 ± 0.0	49.1 ± 0.7
14	[^{18}F]FPy	Si-60	5% EtOAc in cyclohexane	15.2 ± 4.0	-
15	[^{18}F]CPFPX	Si-60	40% EtOAc in cyclohexane	50.8 ± 0.3	-

All RCPs are indicated as mean±SD from experiments performed in triplicate. Abbreviations: s.o.p.: summed integrals of all peaks, p.c.i.: post-column injection. [a] Chromatographic conditions (A = H_2O or H_2O + 0.1% TFA and B = MeCN or MeCN + 0.1% TFA as indicated): [^{18}F]FPy: Chromolith® SpeedROD, gradient: 0–3 min: 2% B, 3–20 min: 8% B, flow rate: 2 mL/min; MultoKrom® and Sunfire®, gradient: 0–5 min: 5% B, 5–20 min: 20% B, flow rate: 1.5 mL/min; Si-60: 5% EtOAc in cyclohexane. [^{18}F]CPFPX: Chromolith® SpeedROD, gradient: 0–3 min: 2% B, 3–20 min: 33% B, flow rate: 2 mL/min; MultoKrom® and Sunfire®, gradient: 0–5 min: 5% B, 5–20 min: 48% B, flow rate: 1.5 mL/min; Si-60: 40% EtOAc in cyclohexane. [b] 52% B (isocratic).

Preliminary experiments showed that the [^{18}F]fluoride peaks with TFA-containing mobile phase were often extremely broad and, in some cases, even overlapped with product peaks, so that a two-step gradient had to be performed to accurately quantify [^{18}F]F$^-$. To this end, [^{18}F]fluoride was allowed to elute during several column volumes under highly aqueous conditions followed by further elution at a solvent strength suitable for analysis of the radiofluorinated compound (Fig. 2), which resulted in significantly increased analysis times.

Merck Chromolith® RP-18 is a stationary phase reported to exhibit low retention of [^{18}F]fluoride [3,6], which is in line with the results of the present experiments. Thus, conventional analysis of the chromatograms obtained with Chromolith® SpeedROD columns using total activities determined by the sum of peaks gave an acceptable accuracy under neutral and acidic conditions. These results were comparable to those obtained using post-column injection (entries 1–4 in table 1). Unfortunately, only short columns with this monolithic stationary phase, which have relatively low separation efficiencies, are commercially available. Thus, for the SpeedROD column applied in this work, the number of theoretical plates (N) determined for the [^{18}F]CPFPX peak (k'=7) amounted to 2300. This performance is sufficient for simple separation tasks, but could be unsatisfactory for analysis of more complex samples.

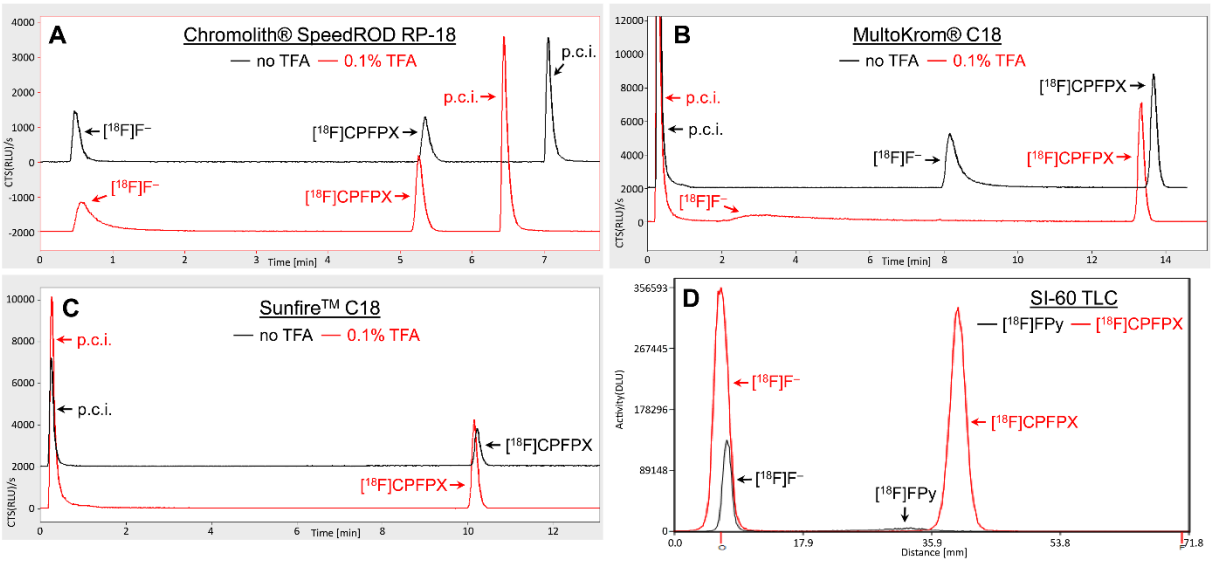

Figure 2. *Representative radio-HPLC and radio-TLC chromatograms. Shown are HPLC traces of the [^{18}F]CPFPX test solutions obtained with neutral (black) or TFA-containing (red) mobile phase* (**A**–**C**) *and TLC traces of the [^{18}F]FPy (black) and [^{18}F]CPFPX (red) test solutions* (**D**). *Abbreviations: p.c.i., reference peak from the post-column injection; TLC, thin layer chromatography.*

In contrast, longer C_{18} columns exhibit much better separation efficiencies, as exemplified by the MultoKrom® C18 column, which provided a plate number of 19000 under identical conditions. Another practical advantage of long columns with regard to post-column injection is the larger void time in the order of 1.5–2.5 min. As nothing elutes from the column during this period, the post-column injection can be performed immediately after sample injection. When the total activity was determined as the sum of peaks, reliable quantification of the RCP from chromatograms obtained with MultoKrom® columns was only possible using a mobile phase without TFA. Application of TFA-containing eluents led to pronounced tailing of the [^{18}F]fluoride peak with up to 10 min peak width (Fig. 2B). As a consequence, integration of the [^{18}F]fluoride peak became highly subjective because it was difficult to locate the end of the peak. Attempts to integrate the peak in a way that a horizontal base line was obtained resulted in overestimation of the RCP for both, [^{18}F]FPy and [^{18}F]CPFPX, by 5 and 7%, respectively. It is important to note that in this case, a considerable amount of signal noise may contribute to the area of the [^{18}F]fluoride peak, which artificially inflates apparent [^{18}F]fluoride recovery and

likely distorts the results to a point where quantification is no longer feasible. On the other hand, the peak of the post-column injection showed only slight tailing and could be integrated much more reliably, so that the reference value of 50% was precisely found when the total activity was determined based on post-column injections (entries 5–8 in table 1). Noteworthy, even under isocratic conditions, accurate results were obtained using post-column injection, while a further increase in the deviation from the reference value to 10% (due to increased [^{18}F]fluoride retention at higher concentrations of organic modifier [3]) was observed for the sum-of-peaks analysis (entry 9 in Table 1). In addition, determination of the total activity by post-column injection significantly reduced the overall analysis time, since it eliminated the need for time-consuming elution of [^{18}F]fluoride under highly aqueous conditions.

While the majority of C_{18} reversed phase columns are based on a similar, chemically modified silica carrier, their properties can exhibit significant differences [9], as illustrated by the results obtained with Waters Sunfire® C18 columns. In this case, no [^{18}F]F$^-$ peaks were observed in chromatograms obtained using either neutral or acidic mobile phase (Fig. 2C). Accordingly, the apparent RCP (RCC) was always 100% when the total activity was determined based on the sum of peak integrals, whereas accurate results were obtained based on post-column injection (entries 10–13 in table 1). While the extremely low recovery of [^{18}F]F$^-$ from Waters Sunfire C_{18} columns under neutral conditions is unusual, many silica-based RP-HPLC columns demonstrate appreciable [^{18}F]fluoride adsorption up to 85% under acidic conditions [3], which could lead to significant overestimation of RCCs if they are determined based on the sum of peak integrals. In addition, these errors would be disproportionally more pronounced for reactions with low ^{18}F-incorporation (due to the associated higher amount of unreacted [^{18}F]fluoride in the reaction mixture), while they should have much less impact in the case of reactions with very high labeling yields. This could in turn, e.g., significantly skew the results obtained during optimization studies and essentially prevent reliable identification of the best labeling conditions. In contrast, high [^{18}F]fluoride adsorption does not affect the accuracy of RCC measurements based on post-column injection, which should therefore provide precise results regardless of the degree of ^{18}F-incorporation. In fact, the quantitative adsorption of [^{18}F]fluoride is even beneficial in this case, as there is no corresponding peak that could interfere with the integration of product peaks.

Radio-TLC represents an alternative method to analyze radiofluorination yields and can be used to quantify compounds that do not migrate on a stationary phase. Accordingly, determination of the RCP by radio-TLC on silica is required by e.g. the European Pharmacopeia for the quality control of PET-tracers for which the presence of [^{18}F]fluoride could not be excluded during development and validation of the production process. However, the method is not well-suited for volatile compounds, including commonly used prosthetic groups like [^{18}F]fluorobenzaldehyde, [^{18}F]fluoroiodobenzene or [^{18}F]fluoroethylazide, as exemplified by the remarkably low (and highly variable) apparent RCPs obtained for [^{18}F]FPy (entry 14 in table 1, Fig 2D). Non-volatile compounds like [^{18}F]CPFPX can be well quantified (entry 15 in table 1), but a low separation efficiency (in this example 200 theoretical plates) substantially limits the applicability of the method. Although attempts have been made to combine the RCC obtained by TLC and the RCP obtained by HPLC to compensate for the individual shortcomings of each

method [10], the significant additional experimental effort required to combine both methods renders this approach unattractive for high throughput analyses.

Having established that post-column injection enables a simple and accurate determination of RCCs by HPLC, we next aimed to demonstrate that the method can be used to reliably predict RCYs as a measure of the overall efficiency of a radiosynthetic procedure without isolation of the product. To this end, activity losses (e.g. due to retention on the QMA resin after pre-processing or adsorption at the reactor surface) had to be taken into account. Importantly, these losses could be readily traced by simple activity measurements with a dose calibrator, which should be common practice during optimization studies. We defined the analytical RCY as the product of the RCC and the fraction of starting activity present in the crude product solution (for details see Eq. 1 in section 2.3). Assuming that tracer isolation by, e.g., preparative HPLC or solid-phase extraction (SPE) is not associated with considerable losses of the radiolabeled compound, the analytical RCY thus obtained should provide a good estimate of the isolated RCY. To verify this assumption, we performed three separate one-pot radiotracer syntheses of [^{18}F]FPy and [^{18}F]CPFPX, and compared the corresponding analytical and isolated RCYs as follows. We first measured the RCC of the crude products after dilution of the reaction mixture with water to calculate analytical RCYs and then isolated the products by semi-preparative HPLC to determine isolated RCYs. Besides the starting activity, three additional parameters were determined for calculation of the analytical RCYs, which comprised (i) residual activity on the QMA cartridge (= A_i in Eq. 1), (ii) residual activity in the empty reactor (= A_{ii} in Eq. 1), and (iii) residual activity in the HPLC syringe (= A_{iii} in Eq. 1). All three values could be measured without additional sample preparation steps either during or after the synthesis and were corrected for decay.

As summarized in Table 2 and Fig. 3, the deviation between analytical and isolated RCYs did not exceed 3% and there was a significant positive correlation ($r=0.995$, $p<0.001$) between the two metrics, demonstrating the accuracy of the proposed method. For more complex radiosynthetic procedures (e.g. two-pot syntheses, procedures involving SPE purification of intermediates or formation of volatile radioactive products) it may be more straightforward to transfer the final crude product solution into a separate vial to measure the remaining activity directly. Apart from 2–3% of the starting activity, which was inevitably retained in the HPLC syringe due to the void volume, isolation by HPLC was not associated with appreciable product losses. Consequently, the number of activity measurements required could be further

Table 2: Comparison of RCC, analytical RCY and isolated RCY for [^{18}F]FPy and [^{18}F]CPFPX.

	Activity losses [%]			RCC (p.c.i)	Analytical RCY	Isolated RCY
Synthesis	**QMA**	**Empty reactor**	**Syringe**	**[%]**	**[%]**	**[%]**
[^{18}F]FPy run 1	5.6	11.9	2.0	58	46	43
[^{18}F]FPy run 2	3.7	8.2	2.3	58	49	48
[^{18}F]FPy run 3	1.3	7.0	2.6	62	55	53
[^{18}F]CPFPX run 1	0.1	2.6	2.1	35	34	35
[^{18}F]CPFPX run 2	0.2	4.5	2.5	39	37	36
[^{18}F]CPFPX run 3	0.1	7.1	2.9	27	24	24

All data are corrected for decay to the start of synthesis.

reduced by neglecting or approximating these minor losses and thereby omitting the term A_{iii} from Eq. 1. For the synthesis of [^{18}F]CPFPX, the RCCs obtained by post-column injection

already provided a very good estimation of the isolated RCYs (0–3% deviation) due to negligible losses during [^{18}F]fluoride processing (resulting in low values of A_i in Eq. 1) and low retention of activity in the reaction vial (resulting in low values of A_{ii} in Eq. 1). However, the noticeable losses of [^{18}F]fluoride by QMA-processing and adsorption in the reactor during the synthesis of volatile [^{18}F]FPy underline the importance of an activity balance, as the difference between RCCs and isolated RCYs for this reaction was up to 15%, whereas the analytical RCYs were again virtually identical to the isolated RCYs.

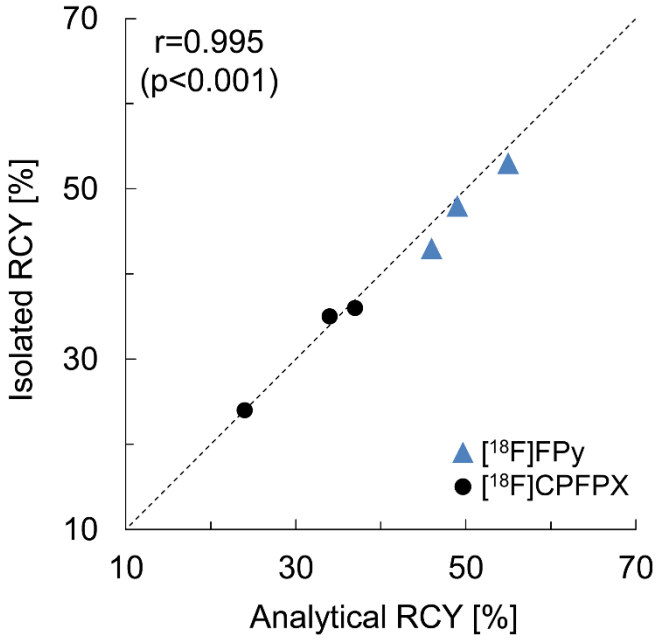

Figure 3. *Correlation between analytical and isolated RCYs for [^{18}F]FPy and [^{18}F]CPFPX. Note that there was a significant (p<0.001) positive correlation (r=0.995) between the two metrics. Dotted line indicates the expected relationship for a perfect correlation between both metrics (e.g. r=1.0).*

4. Conclusion

Analytical HPLC with post-column injection enables precise quantification of the total activity in a sample independent of the chromatographic conditions and therefore provides more reliable RCCs than the conventional sum-of-peaks approach. Furthermore, together with an easily acquired activity balance, post-column injection can be used for rapid and accurate prediction of isolated RCYs, which should substantially improve and accelerate process development in PET-chemistry.

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Graphical Abstract: