



# Determination of Total Radiochemical Purity of [ $^{18}\text{F}$ ]FDG and [ $^{18}\text{F}$ ]FET by High-Performance Liquid Chromatography Avoiding TLC Method

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## Abstract

The goal of this work was to present two high-performance liquid chromatography (HPLC) method that could be applied for the determination of the total radioactive purity of 2-deoxy-2- $^{18}\text{F}$ fluoro-D-glucose ( $^{18}\text{F}$ ]FDG) and *O*-(2- $^{18}\text{F}$ fluoroethyl)-L-tyrosine ( $^{18}\text{F}$ ]FET). The separation of  $^{18}\text{F}$ fluoride ions,  $^{18}\text{F}$ ]FET and  $^{18}\text{F}$ ]FET intermediate was accomplished on LiChrosper RP-18, 250×4 mm, 5  $\mu\text{m}$  (Merck) analytical column. For mobile phase 10 mM potassium dihydrogen phosphate buffer at pH7 (A) and acetonitrile (B) was used: 0–2 min: 15% B; 2–12 min: 85% B; 12–15 min: 15% B, respectively. Analysis of  $^{18}\text{F}$ ]FDG was performed using LiChrosper 100  $\text{NH}_2$ , 250×4.5 mm, 5  $\mu\text{m}$  (Merck) analytical column. The initial mobile phase composition was 10 mM  $\text{KH}_2\text{PO}_4$  buffer (pH7) and acetonitrile (15:85, v/v) and the acetonitrile ratio was decreased to 15% at 2 min after the sample injection and held for 5 min. Complete elution of  $^{18}\text{F}$ fluoride ions from stationary phases could be achieved by adding 10 mg/mL  $\text{K}^{19}\text{F}$  to radioactive samples in a ratio 1:1 during the sample preparation. Recovery of  $^{18}\text{F}$ fluoride ions ranged from 99.5 to 100.6%. The validation of the developed methods showed good results for linearity ( $r^2 = 0.9981\text{--}0.9996$ ), specificity ( $R_S = 3.7\text{--}10.2$ ), repeatability (%Area RSD% = 1.2–4.3%) and limit of quantitation (LOQ = 1.6–4.5 kBq). During the cross-validation similar radiochemical purity values were obtained by the novel HPLC methods and thin layer chromatography performed according to the recommendations of the Ph. Eur. monographs.

**Keywords**  $^{18}\text{F}$ ]FDG ·  $^{18}\text{F}$ ]FET · Quality control · HPLC · Radiochemical purity · PET

## Introduction

Positron emission tomography (PET) is a frequently used imaging technique in the clinical diagnosis of cancer. Substances labeled with radioactive isotopes are used as radiotracers for PET examinations to visualize pathophysiological processes. Predominantly,  $^{18}\text{F}$  isotope is applied for the production of radiopharmaceuticals for human use. The workhorse of PET imaging is  $^{18}\text{F}$ ]FDG, which is used in the assessment of the majority of malignant tumors. Beyond  $^{18}\text{F}$ ]FDG several  $^{18}\text{F}$ -radiopharmaceuticals are available for PET examinations. For instance,  $^{18}\text{F}$ ]fluorocholine is applied for the diagnosis of prostate cancer. Examination

of brain tumors could be performed using  $^{18}\text{F}$ ]FET.  $^{18}\text{F}$ ] fluoromisonidazole could be applied in the assessment of tumor hypoxia [1].

The synthesis of  $^{18}\text{F}$ -labeled radiopharmaceuticals is basically performed using one- or two-step procedure. Radiofluorination of precursors bearing sulfonate leaving groups proceeds via  $\text{S}_{\text{N}}2$  nucleophilic substitution mechanism in polar aprotic solvents. The active pharmaceutical ingredient could be obtained by hydrolysis of the intermediate radioactive compound containing protective functional groups. As a rule, the final solution beside the main component might contain free  $^{18}\text{F}$ fluoride ions and the intermediate compound. The amount of these impurities fundamentally determine the radiochemical purity of the final product [2].

The application of radiopharmaceutical batches for human PET examinations could be started after the determination of radiochemical purity, which is a critical parameter of the quality control system. For this purpose, the separation of radiochemical components and assessment of their radioactivity values are generally performed by

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chromatographic methods. Several high-performance liquid chromatography procedures could be found in the literature for PET radiopharmaceuticals. In the case of [ $^{18}\text{F}$ ]FET, mainly reverse stationary phase (RP) as well as biner mixtures of buffer solutions and organic solvents are applied for the determination of radiochemical purity [3–10]. Orlovskaya et al. demonstrated that using X-Bridge C18 HPLC column (Waters) and applying gradient elution with 0.1% TFA and acetonitrile mixture [ $^{18}\text{F}$ ]FET and [ $^{18}\text{F}$ ]fluorinated intermediate could be successfully separated [3]. On the other hand, Mueller et al. using the same eluent mixture and LiChrospher RP-18e stationary phase (Merck) could not identify unreacted [ $^{18}\text{F}$ ]fluoride in the purified final solution [4]. Wang et al. reported that besides the main [ $^{18}\text{F}$ ]FET peak one unidentified radiochemical impurity could be detected applying Gemini C18 column (Phenomenex) with an eluent of 12% Ethanol and 88% 50 mM  $\text{NaH}_2\text{PO}_4$  buffer (pH 6.8) [5]. In the case of [ $^{18}\text{F}$ ]FDG, mostly ion chromatography is used for the determination of radiochemical purity [11]. Due to this method [ $^{18}\text{F}$ ]fluoride ions and 2-deoxy-2- $^{18}\text{F}$ -fluoro-D-mannose could be separated from the active pharmaceutical ingredient. At the same time, acetylated [ $^{18}\text{F}$ ]FDG, which is the radioactive intermediate compound of [ $^{18}\text{F}$ ]FDG synthesis, undergoes hydrolysis immediately under basic elution conditions [12]. Nakao et al. proposed a novel HPLC method based on aminopropyl-modified silica column and applying acetonitrile and water mixture to detect partially hydrolyzed [ $^{18}\text{F}$ ]FDG [13]. Additionally, the elution of [ $^{18}\text{F}$ ]fluoride ions could be achieved only by decreasing acetonitrile content from the initial 85 to 30%. However, simultaneous separation and detection of [ $^{18}\text{F}$ ]FDG, [ $^{18}\text{F}$ ]fluoride ions and acetylated [ $^{18}\text{F}$ ]FDG could be successfully performed using Rezex columns (Phenomenex) eluted with HPLC grade water [14, 15].

The European Pharmacopoeia (Ph. Eur.) monographs of [ $^{18}\text{F}$ ]FDG and [ $^{18}\text{F}$ ]FET include both HPLC and thin-layer chromatography (TLC) methods for the determination of total radiochemical purity of radiopharmaceuticals [16, 17]. In the case of [ $^{18}\text{F}$ ]FDG, the use of anion-exchange chromatography is recommended. However, the hydrolysis of acetylated [ $^{18}\text{F}$ ]FDG is occurred while using alkaline eluent. On the other hand, [ $^{18}\text{F}$ ]fluoride ions could be hardly detected in the case of [ $^{18}\text{F}$ ]FET analysis. Consequently, additional TLC methods are required to use for the detection of [ $^{18}\text{F}$ ]impurities and the determination of the total radiochemical purity of samples. However, the application of multiple analytical procedures increases the workload and risk of quality control management. To overcome the emerged problem, this work is aimed to develop novel HPLC methods for the determination of total radiochemical purity of [ $^{18}\text{F}$ ]FDG and [ $^{18}\text{F}$ ]FET avoiding TLC methods. Hydrophilic interaction liquid chromatography (HILIC) and RP-HPLC are tested for the separation of radiochemical

compounds. Since the analysis of [ $^{18}\text{F}$ ]fluoride ions in radiopharmaceutical samples is a great challenge for radioanalytical chemists [18], special emphasis is given to elution profile optimization and investigation of the recovery of [ $^{18}\text{F}$ ]fluoride ions.

## Experimental

### Chemicals

Acetonitrile was supplied from VWR. Sodium hydroxide, potassium fluoride, potassium dihydrogen phosphate and phosphoric acid were obtained from Sigma Aldrich. Fluoroethyl-L-tyrosine, 3,4-dimethoxy-L-phenylalanine, 2-deoxy-2-fluoro-D-glucose, 2-deoxy-2-chloro-D-glucose and 2-deoxy-2-fluoro-D-mannose was purchased from ABX (Radeberg, Germany). All chemicals used for the experiments were HPLC grade and applied without additional purification. Water used for dilution was provided by a Milli-Q purification system and controlled for the content of organic impurities.

### Preparation of Radioactive Standards

#### $\text{H}^{18}\text{F}$ Fluoride

Production of  $^{18}\text{F}$  isotope was performed by irradiation of enriched water (1.5 mL,  $\geq 95\%$   $\text{H}_2^{18}\text{O}$ ), Rotem Industries Ltd., Israel) in niobium target for up to 120 min. GE PETtrace cyclotron was used for this purpose with 70  $\mu\text{A}$  of 16 MeV protons. Typically, 1–200 GBq of total radioactivity was produced by the  $^{18}\text{O}(p,n)^{18}\text{F}$  nuclear reaction. The obtained  $\text{H}^{18}\text{F}$  solution was transferred remotely into the radiochemical hot cell by helium gas push (99.9999% purity, Linde) through 1/8 in. PEEK tube either for direct use in HPLC method development or for further radiochemical transformation.

#### $^{18}\text{F}$ FDG and Acetylated $^{18}\text{F}$ FDG

The Hamacher process was applied for the synthesis of [ $^{18}\text{F}$ ]FDG and the intermediate product [19]. [ $^{18}\text{F}$ ]fluoride ions were extracted from target water onto SepPak Light QMA Cartridge (Waters) and subsequently eluted with a mixture of 15 mg Kryptofix 222 (Merck) in 0.8 mL acetonitrile (Sigma) and 3.5 mg  $\text{K}_2\text{CO}_3$  (Sigma) in 0.25 mL water. The effluent was delivered to the reaction vessel and azeotropically evaporated to dryness. In the next step, 20 mg of 1,3,4,6-tetra-*O*-acetyl-2-*O*-trifluoromethanesulfonyl- $\beta$ -D-mannopyranose precursor (mannose triflate, ABX Advanced Biochemical Compounds, Germany) in 1 mL of anhydrous acetonitrile was added to the reaction vessel. The

nucleophilic substitution reaction was carried out in 1 min at 85 °C. The produced acetylated [<sup>18</sup>F]FDG was used directly for chromatographic method optimization or for further synthesis of [<sup>18</sup>F]FDG which was proceeded with the evaporation step and subsequent removal of protective acetyl groups by hydrolysis with HCl (1 M, 1 mL) in 3.5 min at 121 °C. C18 Plus and Alumina N SepPak Cartridges (Waters) were used for the purification of the reaction mixture. The pH was adjusted using AG11A8 ion retardation resin (Bio-Rad). The purified solution was diluted with 14 mL water. Finally, the solution was filtered through 0.22 µm Millex-GS Syringe-Driven Filter Unit (Millipore). The synthesis time was 23 min and up to 130 GBq radioactivity was obtained. The production of [<sup>18</sup>F]FDG and the intermediate compound was accomplished by an automated GE Tracerlab FX-FDG synthesizer.

### [<sup>18</sup>F]FET and [<sup>18</sup>F]FET Intermediate

The production of [<sup>18</sup>F]FET and [<sup>18</sup>F]FET intermediate was performed on Scintomics GRP 3 V synthesis module applying ready-to-use Ph. Eur. compliant kits obtained from ABX (Radeberg, Germany). The synthesis was performed via nucleophilic substitution reaction between [<sup>18</sup>F]fluoride ions and (2S)-O-(2'-tosyloxyethyl)-N-trityl-tyrosine-tert-butyl ester (TET) precursor [8]. [<sup>18</sup>F]fluoride ions were harvested from target water using SepPak Light QMA Cartridge and the subsequent elution of the radioactivity was accomplished with 0.075 mol/L tetrabutylammonium-bicarbonate solution. The mixture was azeotropically dried and 10 mg of the precursor was added in 2 mL acetonitrile to the reactor. The substitution was performed at 110 °C in 5 min. The produced [<sup>18</sup>F]FET intermediate was used directly for HPLC method development or for further synthesis of [<sup>18</sup>F]FET which proceeded with the evaporation of acetonitrile and subsequent removal of protective groups by hydrolysis with HCl (0.2 M, 3 mL) in 8 min at 120 °C. SepPak Light Alumina N, SepPak WAX and SepPak HLB Cartridges were used for purification of the raw reaction mixture. The product was eluted from cartridges with 12 mL ethanol and water mixture (1:19, v/v). Finally, the product was filtered through 0.22 µm Millex-GS Syringe-Driven Filter Unit (Millipore). The synthesis time was 55 min and up to 50 GBq radioactivity was obtained.

### Instrumentation

The HPLC analyses were conducted on a Jasco (Japan) liquid chromatograph system which included a PU-2080 HPLC pump, AS-950-10 autosampler, LG-2080-04 quaternary gradient unit. <sup>18</sup>F-labeled compounds were detected by a radioactive detector (NaI (TI)/PMT, Hamamatsu). The detection of non-radioactive ingredients was performed by

UV-2075 and RI-2031 (Jasco, Japan) detectors. Data acquisition and signal processing were accomplished using LC-Net II/ADC interface and ChromNav Software. Separation was performed on LiChrosper 100 NH<sub>2</sub>, 250×4.5 mm, 5 µm (Merck) and LiChrosper RP18, 250×4 mm, 5 µm (Merck) at ambient temperature.

Thin-layer chromatography measurements were carried out on miniGita Star device supplied with a GMC counting tube and controlled by miniGita control software (raytest).

### Methods

The analysis of [<sup>18</sup>F]FET samples according to the recommendation of Ph. Eur. monograph [17] was performed using the following reverse phase HPLC method. LiChrosper RP-18, 250×4 mm, 5 µm (Merck) was applied for the stationary phase. The elution was performed using carbon dioxide-free water (A) and acetonitrile (B) for the mobile phase. The separation was accomplished according to the following gradient steps. At the beginning, the content of mobile phase A was 90% and at the point of 10 min it was started to reduce to 5% A within 10 min and held for a further 10 min. The injected volume was 20 µL and the flow rate was adjusted to 1 mL/min. The retention time of [<sup>18</sup>F]fluoride ions, [<sup>18</sup>F]FET and [<sup>18</sup>F]FET intermediate was about 1.5, 6.7 and 9.3 min, respectively.

The following anion-exchange HPLC method was used for the determination of radiochemical purity of [<sup>18</sup>F]FDG which was in compliance with Ph. Eur. monograph [16]. Dionex CarboPack PA10 250×4 mm, 10 µm (Thermo Scientific) was applied for the separation of radiochemical components. For the mobile phase 0.1 mmol/L sodium hydroxide was used at 1 mL/min flow rate. The injected volume was 20 µL. The retention time of [<sup>18</sup>F]fluoride ions and [<sup>18</sup>F]FDG was about 4.7 and 9.8 min, respectively.

The determination of radiochemical purity of [<sup>18</sup>F]FDG and [<sup>18</sup>F]FET samples was accomplished by thin-layer chromatography based on the European Pharmacopoeia monographs as follows [16, 17]. Silica gel 60 plate (Merck) was employed as the stationary phase. A mixture of acetonitrile and water (95:5, v/v) was applied as a mobile phase in the analysis of [<sup>18</sup>F]FDG. In the case of [<sup>18</sup>F]FET methanol and 300 g/L acetic acid (90:10, v/v) eluent was used. 1 µL of the sample was applied to the TLC plate and developed over a path of 8 cm and 14 cm for [<sup>18</sup>F]FDG and [<sup>18</sup>F]FET, respectively. After the development, the plate was allowed to dry in the air for 10 min. The radioactivity ratio of the <sup>18</sup>F-labeled components was determined as %Area of the region of interest (ROI) by miniGita Star TLC scanner (raytest). On the one hand, the retention factor ( $R_f$ ) of [<sup>18</sup>F]fluoride ions, [<sup>18</sup>F]FDG and acetylated [<sup>18</sup>F]FDG were about 0, 0.4 and 0.8, respectively. On the other hand, the  $R_f$  of [<sup>18</sup>F]fluoride ions,

[ $^{18}\text{F}$ ]FET and [ $^{18}\text{F}$ ]FET intermediate was about 0, 0.6 and 0.7, respectively.

The development of novel HPLC methods for the determination of total radiochemical purity of [ $^{18}\text{F}$ ]FDG and [ $^{18}\text{F}$ ]FET was accomplished using LiChrosper RP-18, 250 × 4 mm, 5  $\mu\text{m}$  (Merck) and LiChrosper 100  $\text{NH}_2$ , 250 × 4.5 mm, 5  $\mu\text{m}$  (Merck) analytical columns. For the mobile phase 10 mM potassium dihydrogen phosphate buffer at pH7 (A) and acetonitrile (B) was used to perform the optimized elution profile. Eluent A was obtained by dissolution of 1.36 g  $\text{KH}_2\text{PO}_4$  in water and then 4.7 mL of 1.0 M sodium hydroxide solution was added to the mixture which was diluted to 1000 mL with the same solvent. The prepared mobile phase was filtered through Supelco membrane filter of 0.45  $\mu\text{m}$  pore size. Chemical ingredients of [ $^{18}\text{F}$ ]FET sample were detected at 225 nm wavelength. Non-radioactive compounds in [ $^{18}\text{F}$ ]FDG were identified by a refractive index detector. Chromatographic measurements were carried out at ambient temperature. The injected volume was 20  $\mu\text{L}$  and the flow rate was adjusted to 1 mL/min.

### Sample Preparation

Solutions of radioactive reference compounds extracted from cyclotron or automated synthesizer were directly injected into HPLC system or applied to TLC plate. During the investigation of recovery of [ $^{18}\text{F}$ ]fluoride ions 10 mg/mL potassium fluoride solution was added to the radioactive sample with 1:1 ratio. The radioactivity values of samples for the validation procedure were adjusted by dilution with water or by injection of samples at a different time interval. The radioactivity concentration of the samples was up to 1 GBq/mL. The measurements were performed in triplicate despite linearity tests.

## Results and Discussion

### HPLC Method Development for Analysis of [ $^{18}\text{F}$ ]FET

The separation and detection of identified radiochemical components of [ $^{18}\text{F}$ ]fluoride ions, [ $^{18}\text{F}$ ]FET and [ $^{18}\text{F}$ ]FET intermediate is crucial for the determination of the total radiochemical purity of the radiopharmaceutical sample. For this purpose, the authors have applied a high-performance liquid chromatography method in accordance with the recommendation of the European Pharmacopoeia monograph [17]. Despite the fact that [ $^{18}\text{F}$ ]fluoride ions could be eluted from LiChrosper RP-18 column using gradient elution with water and acetonitrile mixture, a poor peak shape was obtained and the recovery was less than 85%. This is the reason why an additional TLC method is usually applied for the determination of the total radiochemical purity of

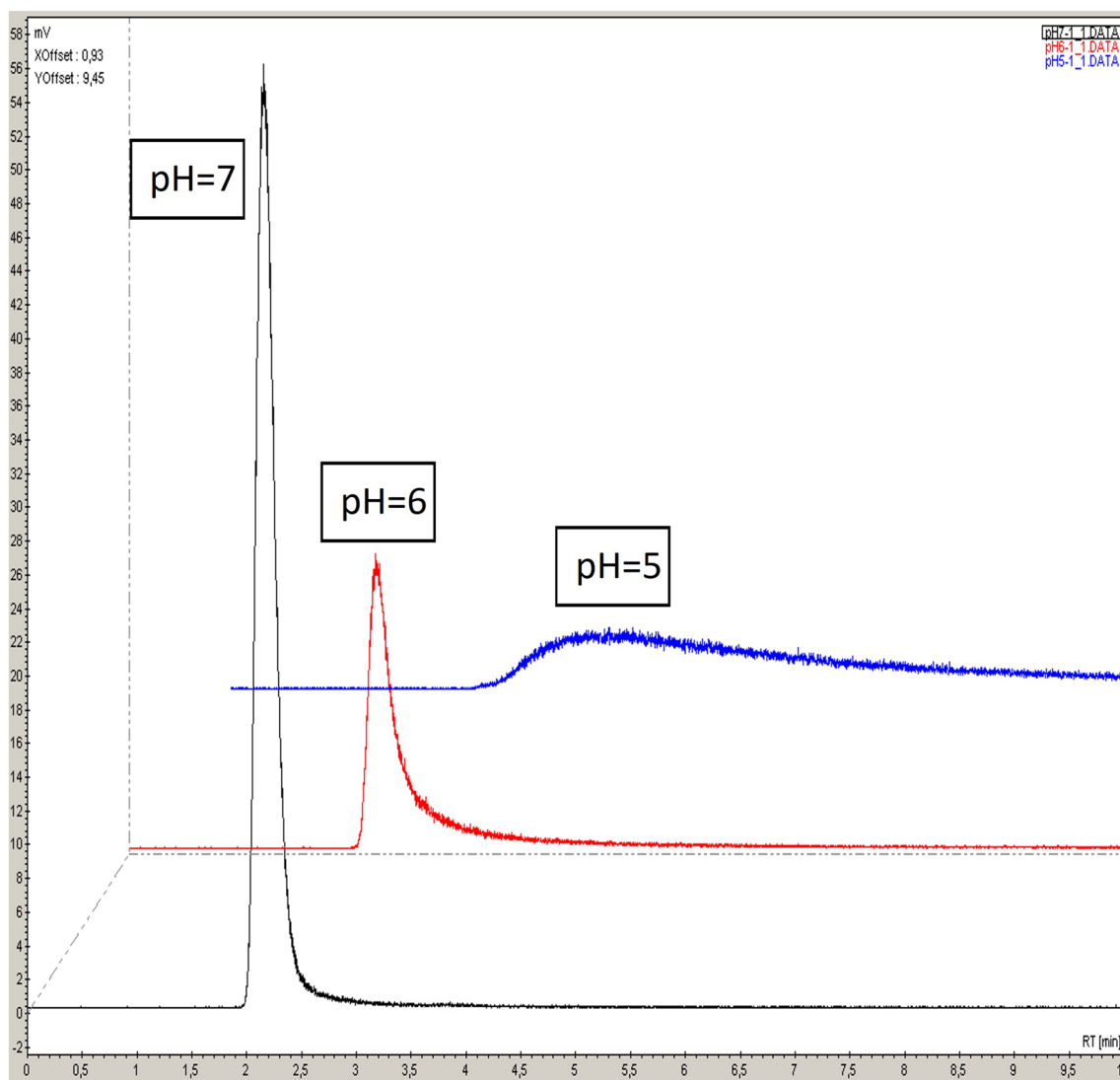
radiopharmaceuticals. To overcome this problem in this work the optimization of chromatographic conditions was carried out for the development of an HPLC method for analysis of [ $^{18}\text{F}$ ]FET and avoiding TLC procedure.

### Optimization of Mobile Phase pH for Effective Elution of [ $^{18}\text{F}$ ]Fluoride Ions

The retention and peak shape of [ $^{18}\text{F}$ ]fluoride ions performing RP-HPLC analysis highly depends on the pH of the buffer component of the eluent [18]. As a first step of the method development, the effect of pH of mobile phase was examined on elution parameters of [ $^{18}\text{F}$ ]fluoride ions using LiChrosper RP-18, 250 × 4 mm, 5  $\mu\text{m}$  (Merck) analytical column. 10 mM buffer solution of potassium dihydrogen phosphate was used as eluent A and acetonitrile as eluent B. The elution was accomplished according to the following gradient profile. At the beginning mobile phase A ratio was 85% and at the point of 10 min after the injection it was started to reduce to 15% within 10 min and held for further 10 min. The injected volume was 20  $\mu\text{L}$  and the flow rate was adjusted to 1 mL/min. The pH of phosphate buffer was adjusted to 3, 4, 5, 6 and 7. After the analysis of samples, no chromatographic peak was detected at pH 3 and 4. Apparently, [ $^{18}\text{F}$ ]fluoride ions were adsorbed on column material in these cases since the  $\text{pK}_a$  is 3.17 for hydrogen fluoride and the ion suppressed form of [ $^{18}\text{F}$ ]fluoride has a higher affinity to the stationary phase. At pH5 a broad peak was appeared at about 5 min retention time and with a tailing factor of 3.2 (Fig. 1). Increasing the pH of the buffer solution to 6 and 7 the retention time was decreased to 3.3 and 2.2 min and the tailing factor decreased to 2.1 and 1.3, respectively. Consequently, it is recommended to adjust the pH of the buffer component of the mobile phase to 7 for effective elution of [ $^{18}\text{F}$ ]fluoride ions from the stationary phase.

### Enhancement of [ $^{18}\text{F}$ ]Fluoride Ions Recovery by Co-injection with $\text{K}[^{19}\text{F}]$

It is well known from the literature, that [ $^{18}\text{F}$ ]fluoride ions tend to retain in the stationary phase at appropriate pH [18]. However, complete elution of radioactivity from the HPLC system is important for accurate determination of the radiochemical purity. In our case, the recovery of [ $^{18}\text{F}$ ]fluoride ions was assessed by comparison of decay corrected peak areas obtained from the analysis of samples injected onto the analytical column and a procedure in which the sample bypassed the HPLC column and flowed directly through the radioactivity detector [20]. In accordance with the Ph. Eur. recommendations, using water and acetonitrile mixture for mobile phase the obtained [ $^{18}\text{F}$ ]fluoride recovery was less than 85% (Table 1). At the same time, this value was increased up to 94.7% while using 10 mM  $\text{KH}_2\text{PO}_4$  buffer



**Fig. 1** Effect of pH of mobile phase on the elution of [<sup>18</sup>F]fluoride ions from LiChrosper RP-18 column

**Table 1** Influence of K[<sup>19</sup>F] and the composition of mobile phase on the recovery of [<sup>18</sup>F]fluoride ions

<i>n/n</i>	<i>c</i> (K[ <sup>19</sup> F]), mg/mL	Eluent A	Recovery, %
1	–	Water	84.0 ± 0.5
2	10	Water	99.6 ± 2.4
3	–	10 mM KH <sub>2</sub> PO <sub>4</sub> buffer (pH7)	94.7 ± 1.6
4	10	10 mM KH <sub>2</sub> PO <sub>4</sub> buffer (pH7)	99.5 ± 1.1

(pH7). Presumably, the increase of ion strength of the mobile phase enhanced the recovery of [<sup>18</sup>F]fluoride ions. Additionally, the measured [<sup>18</sup>F]fluoride recovery values could be increased to a maximum by adding 10 mg/mL K[<sup>19</sup>F] solution to the radioactive sample in 1:1 ratio. Namely, using

water or phosphate buffer for eluent A due to the stable fluoride carrier the recovery was 99.6 and 99.5%, respectively. Consequently, complete elution of [<sup>18</sup>F]fluoride ions could be achieved by adding potassium fluoride to the measured radiopharmaceutical sample.

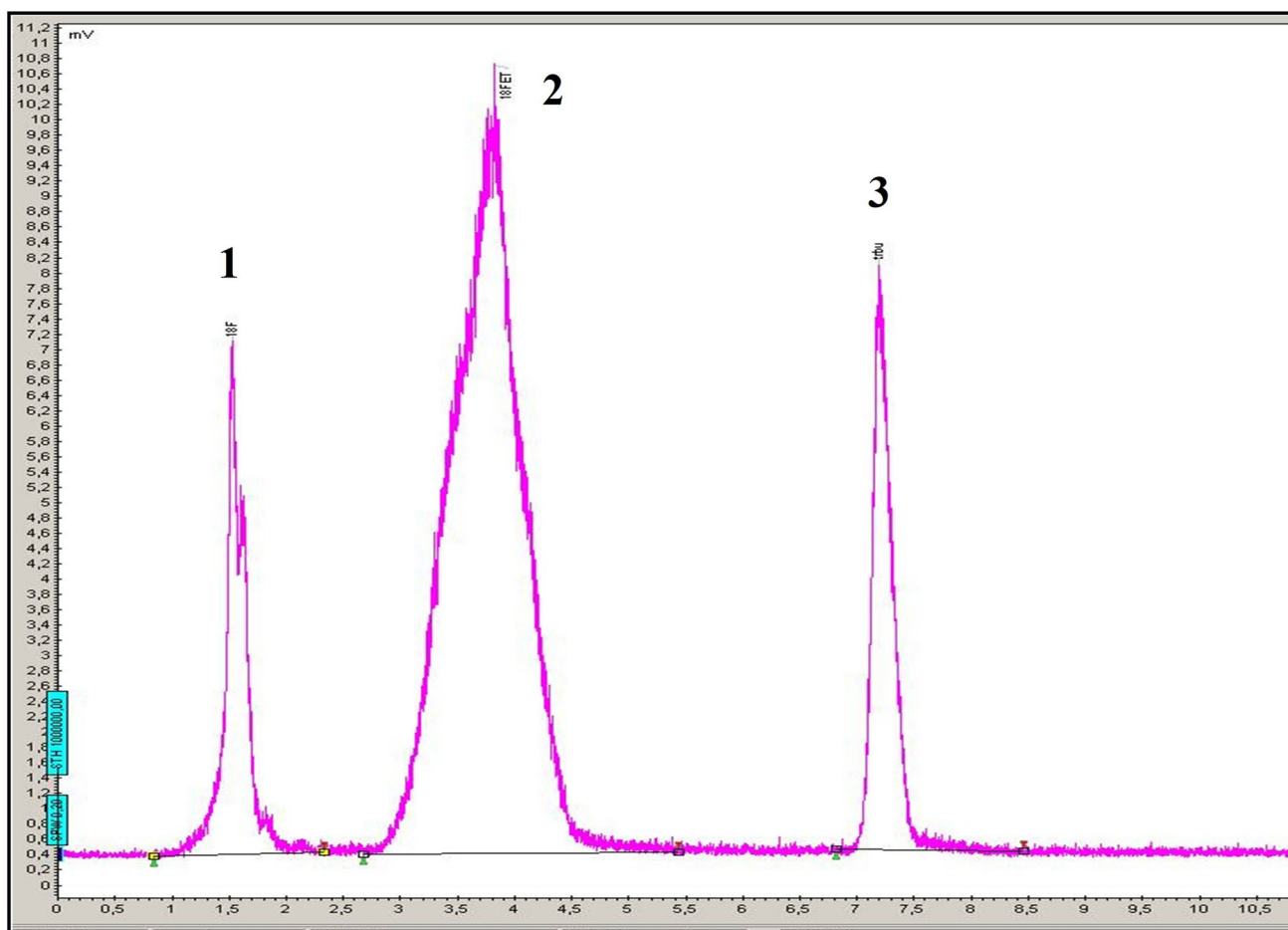
### Gradient Elution for Determination of Radiochemical Purity of [<sup>18</sup>F]FET

According to our results LiChrosper RP-18, 250 × 4 mm, 5 μm (Merck) analytical column could be successfully applied for the separation of radiochemical ingredients of [<sup>18</sup>F]FET. On the other hand, the composition of the mobile phase had to be optimized for elution of [<sup>18</sup>F]fluoride ions, [<sup>18</sup>F]FET and [<sup>18</sup>F]FET intermediate from the stationary phase within acceptable measurement time. Based on the

previous results of [ $^{18}\text{F}$ ]fluoride recovery study a gradient elution was developed for the analysis of [ $^{18}\text{F}$ ]FET. 10 mM potassium dihydrogen phosphate buffer at pH7 (A) and acetonitrile (B) were used as mobile phase. The initial mobile phase A content was adjusted to 85% to obtain maximum recovery for [ $^{18}\text{F}$ ]fluoride which was eluted at about 1.5 min after the sample injection (Fig. 2). After 2 min of the injection, the ratio of acetonitrile was changed to 85% and held for 10 min. The elution of [ $^{18}\text{F}$ ]FET and [ $^{18}\text{F}$ ]FET intermediate was accomplished with a retention time of 3.7 and 7.1 min, respectively. The resolution ( $R_s$ ) of [ $^{18}\text{F}$ ]fluoride and [ $^{18}\text{F}$ ]FET as well as [ $^{18}\text{F}$ ]FET and [ $^{18}\text{F}$ ]FET intermediate was 3.7 and 5.7, respectively. The measurement time was 15 min.

After the optimization of mobile phase composition, the developed method was validated. Linearity was verified in the radioactivity concentration range of 42–991 MBq/mL (Supplementary information, Fig. S1–S3). The obtained regression coefficients ( $r^2$ ) were 0.9996. The repeatability was determined by injection of 6 replicates of reference solution containing the radiochemical ingredients. The relative

standard deviation (RSD%) of %Area for [ $^{18}\text{F}$ ]fluoride ions, [ $^{18}\text{F}$ ]FET and [ $^{18}\text{F}$ ]FET intermediate was 3.6, 1.2 and 2.8%, respectively. The limit of quantitation of [ $^{18}\text{F}$ ]fluoride ions and [ $^{18}\text{F}$ ]FET intermediate was 1.6 and 2.9 kBq, respectively. Identification of [ $^{18}\text{F}$ ]FET was performed by analysis of radiopharmaceutical sample spiked with non-radioactive fluoroethyl-L-tyrosine solution with a concentration of 10  $\mu\text{g/mL}$ . The relative difference between retention times of [ $^{18}\text{F}$ ]FET and [ $^{19}\text{F}$ ]FET peaks was in the range of 1.1 and 3.5%. The specificity was evaluated as peak resolution between [ $^{18}\text{F}$ ]fluoride ions and [ $^{18}\text{F}$ ]FET as well as [ $^{18}\text{F}$ ]FET and [ $^{18}\text{F}$ ]FET intermediate which ranged from 3.7 to 5.7. Robustness was examined at small changes of optimal parameters of flow rate, eluent ratio, pH and ionic strength of mobile phase. The obtained peak resolutions of [ $^{18}\text{F}$ ]compounds were in the range of 2.3 and 6.7 (Supplementary information, Table S1). The cross validation was performed by comparing the radiochemical purities of three [ $^{18}\text{F}$ ]FET batches determined by the novel HPLC method and Ph. Eur. procedures (Table 2). It was found that similar results were obtained by the novel liquid chromatographic method



**Fig. 2** Radiochromatogram of [ $^{18}\text{F}$ ]FET using optimized gradient elution (1: [ $^{18}\text{F}$ ]fluoride ions, 2: [ $^{18}\text{F}$ ]FET, 3: [ $^{18}\text{F}$ ]FET intermediate)

**Table 2** Cross validation of novel HPLC methods and Ph. Eur. procedures

Batch	Radiochemical purity, %		
	HPLC (Ph. Eur.)	TLC (Ph. Eur.)	HPLC (novel)
[ <sup>18</sup> F]FET #1	79.0 ± 0.2	75.7 ± 0.4	75.2 ± 0.1
[ <sup>18</sup> F]FET #2	69.5 ± 0.2	66.3 ± 0.5	66.1 ± 0.1
[ <sup>18</sup> F]FET #3	75.9 ± 0.1	73.1 ± 0.3	72.7 ± 0.2
[ <sup>18</sup> F]FDG #1	93.3 ± 0.1	90.2 ± 0.3	90.8 ± 0.2
[ <sup>18</sup> F]FDG #2	88.8 ± 0.1	85.9 ± 0.4	86.1 ± 0.1
[ <sup>18</sup> F]FDG #3	84.8 ± 0.2	81.1 ± 0.4	80.8 ± 0.2

and thin-layer chromatography. However, the radiochemical purity determined by the HPLC method was performed according to Ph. Eur. monograph was somewhat higher due to partial elution of [<sup>18</sup>F]fluoride ions from the stationary phase.

### Chemical Purity Test of [<sup>18</sup>F]FET

Applying UV detection the developed HPLC method is also eligible for simultaneous determination of chemical purity of [<sup>18</sup>F]FET since the system suitability test could be performed successfully according to the requirements of Ph. Eur. monograph [17]. Analyzing a reference solution of 3,4-dimethoxy-L-phenylalanine and fluoroethyl-L-tyrosine with a concentration of 250 and 50 µg/mL, respectively, the compounds could be separated with a resolution of 4.1 which is higher than the minimum limit of 2.0 (Supplementary information, Fig. S4). During the validation of the chemical purity test linearity was verified in the concentration range of 0.01–300 µg/mL and 0.01–60 µg/mL for 3,4-dimethoxy-L-phenylalanine and fluoroethyl-L-tyrosine, respectively. The obtained regression coefficients ( $r^2$ ) were in the range of 0.9985 and 0.9990. The repeatability of peak area and retention time of components was determined by injection of 6 replicates of the reference solution with a concentration of 250 and 50 µg/mL of 4-dimethoxy-L-phenylalanine and fluoroethyl-L-tyrosine, respectively. The calculated relative standard deviations (RSD%) were in the range from 0.2 to 0.7%. Additionally, intermediate precision was in the range of 0.1 and 1.1% which was obtained from 6 injections of the reference solution performed on different days. The accuracy was determined as a recovery of adjusted 60 µg/mL concentration of FET which ranged from 96.1 to 97.3% ( $n = 6$ ). Limit of quantitation of 3,4-dimethoxy-L-phenylalanine and fluoroethyl-L-tyrosine was 0.02 and 0.05 µg/mL, respectively. Robustness was examined at small changes in optimal parameters of flow rate, eluent ratio, pH and ionic strength of mobile phase. The obtained resolutions of 3,4-dimethoxy-L-phenylalanine and fluoroethyl-L-tyrosine peaks were in the range of 2.8 and 4.6 (Supplementary information, Table S2).

The HPLC analysis of [<sup>18</sup>F]FET batches revealed that no relevant non-radioactive impurities could be detected in samples (Supplementary information, Fig. S5).

### Analysis of [<sup>18</sup>F]FDG by HILIC Method

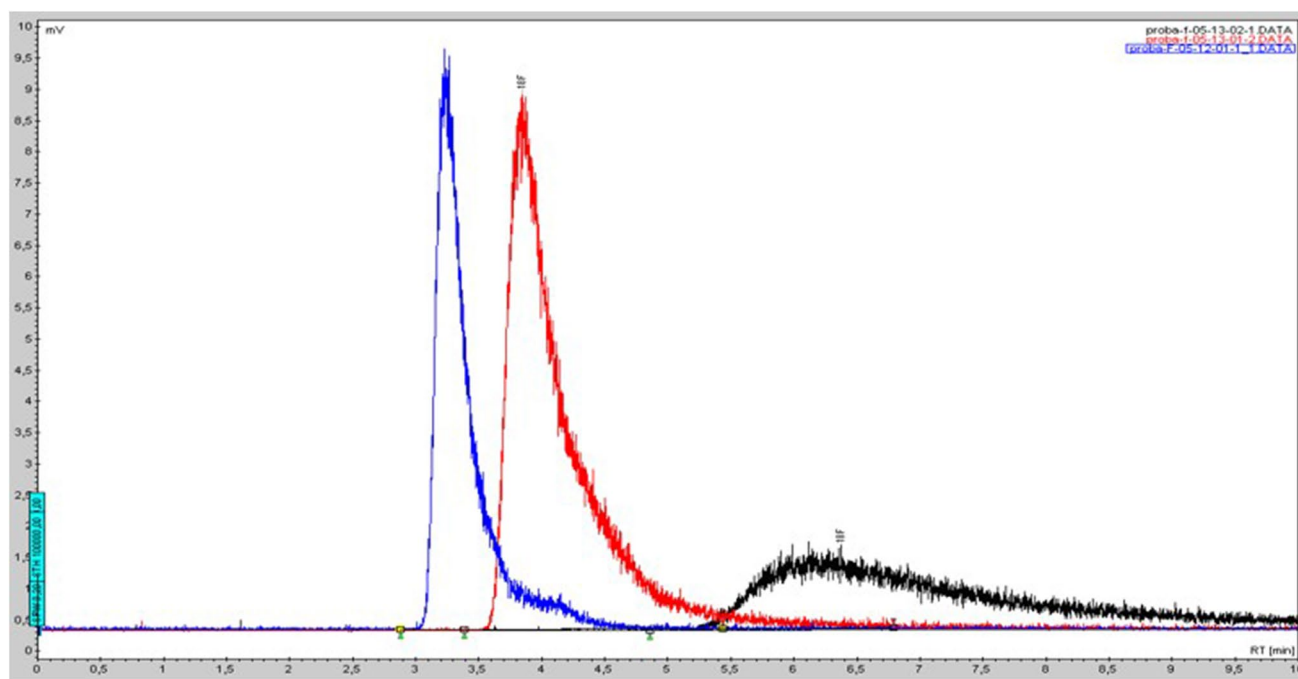
The total radiochemical purity of [<sup>18</sup>F]FDG could not be determined exclusively using anion-exchange HPLC method of the Ph. Eur. monograph due to the hydrolysis of acetylated [<sup>18</sup>F]FDG under the basic chromatographic conditions [16]. Additionally, the authors have found that applying 0.1 mmol/L sodium hydroxide as mobile phase [<sup>18</sup>F]fluoride ions could be eluted from Dionex CarboPack PA10 250 × 4 mm, 10 µm (Thermo Scientific) column with the recovery of up to 97%. To perform the determination of the total radiochemical purity of [<sup>18</sup>F]FDG a novel method was developed based on hydrophilic interaction liquid chromatography.

### Influence of pH of Mobile Phase on the Elution of [<sup>18</sup>F]Fluoride Ions

The HILIC method was developed based on aminopropyl modified LiChrosper 100 NH<sub>2</sub>, 250 × 4.5 mm, 5 µm (Merck) analytical column. The investigation of [<sup>18</sup>F]fluoride elution was a critical part of the chromatographic optimization procedure. Interestingly, using water (A) and acetonitrile (B) mixture as mobile phase in 85:15 ratio at 1 mL/min flow rate was not appropriate for elution of [<sup>18</sup>F]fluoride ions as it was observed in the case of LiChrosper RP-18, 250 × 4 mm, 5 µm (Merck) column. To solve this problem mobile phase A was changed to 10 mM potassium dihydrogen phosphate solution. Measurements were accomplished at isocratic conditions and the injected volume was 20 µL. The pH of the buffer solution was adjusted to 3, 4, 5, 6 and 7. After the injection of samples, it was found that [<sup>18</sup>F]fluoride was not eluted from the column at pH 3 and 4. On the other hand, a broad peak was detected at 6.4 min retention time and with a tailing factor of 3.0 at pH5 (Fig. 3). Increasing the pH of the buffer solution to 6 and 7 the retention time was decreased to 3.8 and 3.2 min and the tailing factor decreased to 2.8 and 2.3, respectively. Consequently, phosphate buffer should be applied instead of water and the pH is recommended to maintain at 7 for effective elution of [<sup>18</sup>F]fluoride ions from the stationary phase.

### Effect of K[<sup>19</sup>F] Additive on the Recovery of [<sup>18</sup>F] Fluoride Ions

The determination of recovery of [<sup>18</sup>F]fluoride ions during the elution from LiChrosper 100 NH<sub>2</sub> stationary phase was accomplished by comparison of decay corrected peak areas obtained from two subsequent measurements in the presence



**Fig. 3** Effect of pH of mobile phase on the elution of [ $^{18}\text{F}$ ]fluoride ions from LiChrosper LiChrosper 100  $\text{NH}_2$  column

and absence of HPLC column [20]. A mixture of 10 mM  $\text{KH}_2\text{PO}_4$  buffer (pH7) and acetonitrile in 85:15 ratio was applied for the analysis, which was the optimal composition of the mobile phase. Interestingly, the measured [ $^{18}\text{F}$ ]fluoride recovery was only 80.4%. This value could be enhanced only by the addition of 10 mg/mL  $\text{K}[^{19}\text{F}]$  solution to the radioactive sample in 1:1 ratio. In this case, the recovery of [ $^{18}\text{F}$ ]fluoride ions increased up to 100.6%. Consequently, the addition of non-radioactive potassium fluoride to the sample is necessary for the complete elution of [ $^{18}\text{F}$ ]fluoride ions.

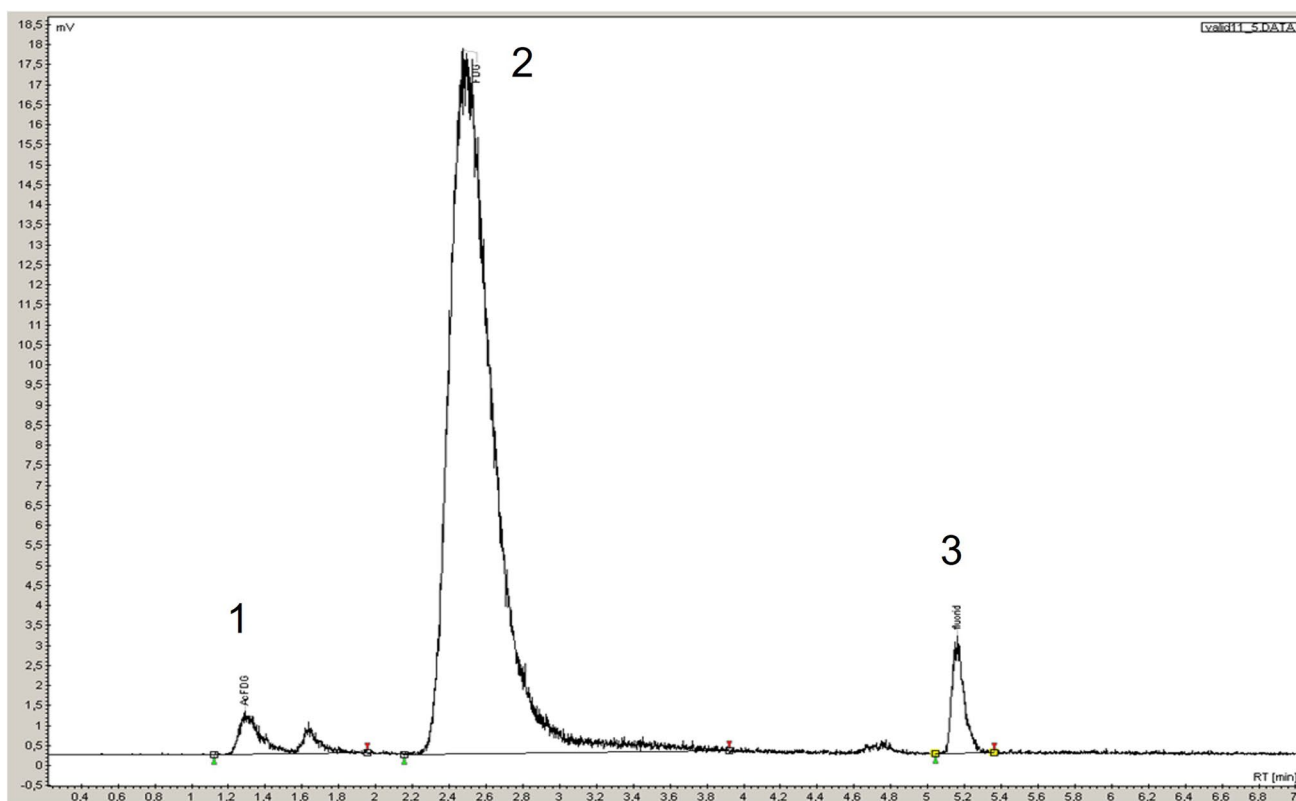
### Analysis of [ $^{18}\text{F}$ ]FDG Using HILIC Method

The radiochemical purity of [ $^{18}\text{F}$ ]FDG was managed to determine using a gradient elution due to the considerable difference in polarity of the radiochemical ingredients, namely [ $^{18}\text{F}$ ]fluoride ions, [ $^{18}\text{F}$ ]FDG and acetylated [ $^{18}\text{F}$ ]FDG. The separation of components was performed on LiChrosper 100  $\text{NH}_2$ , 250  $\times$  4.5 mm, 5  $\mu\text{m}$  (Merck) analytical column using the mixture of 10 mM potassium dihydrogen phosphate buffer at pH7 (A) and acetonitrile (B) as mobile phase. The initial mobile phase B content was adjusted to 85% to elute acetylated [ $^{18}\text{F}$ ]FDG with the lowest polarity and [ $^{18}\text{F}$ ]FDG with a retention time of 1.3 and 2.5 min, respectively (Fig. 4.). The ratio of mobile phase B was changed to 15% at the point of 2 min after the sample injection and hold for 5 min. The retention time of [ $^{18}\text{F}$ ]fluoride ions was 5.1 min. The resolution of acetylated [ $^{18}\text{F}$ ]FDG and [ $^{18}\text{F}$ ]FDG as well as [ $^{18}\text{F}$ ]fluoride and [ $^{18}\text{F}$ ]FDG

peaks was 4.2 and 10.2, respectively. The measurement time was 7 min.

After the optimization of gradient elution, the developed method was validated. Linearity was verified in the radioactivity concentration range of 5–902 MBq/mL (Supplementary information, Fig. S6–S8). The regression coefficients were obtained between 0.9981 and 0.9995. The repeatability was determined by injection of 6 replicates of reference solution containing the radiochemical ingredients. The relative standard deviation of %Area for [ $^{18}\text{F}$ ]fluoride ions, [ $^{18}\text{F}$ ]FET and acetylated [ $^{18}\text{F}$ ]FDG intermediate was 1.5, 2.0 and 4.3%, respectively. The limit of quantitation of [ $^{18}\text{F}$ ]fluoride ions and acetylated [ $^{18}\text{F}$ ]FDG was 1.9 and 4.5 kBq, respectively. Identification of [ $^{18}\text{F}$ ]FDG was performed by analysis of radiopharmaceutical sample spiked with non-radioactive 2-deoxy-2-fluoro-D-glucose solution with a concentration of 1 mg/mL. The relative difference between retention times of [ $^{18}\text{F}$ ]FDG and [ $^{19}\text{F}$ ]FDG peaks was in the range of 1.1 and 3.5%. The peak resolution between acetylated [ $^{18}\text{F}$ ]FDG and [ $^{18}\text{F}$ ]FDG as well as [ $^{18}\text{F}$ ]FDG and [ $^{18}\text{F}$ ]fluoride ions ranged from 4.2 to 10.2. Robustness was examined at small changes in optimal parameters of flow rate, eluent ratio, pH and ionic strength of mobile phase. The obtained peak resolutions of [ $^{18}\text{F}$ ]compounds were in the range of 2.9 and 10.5 (Supplementary information, Table S3). The cross validation was performed by comparing the determined radiochemical purities of three [ $^{18}\text{F}$ ]FET batches by the novel HPLC method and Ph. Eur. procedures (Table 2). It was found that similar results were obtained by the developed





**Fig. 4** Radiochromatogram of [<sup>18</sup>F]FDG obtained by the HILIC method (1: [<sup>18</sup>F]fluoride ions, 2: [<sup>18</sup>F]FDG, 3: acetylated [<sup>18</sup>F]FDG)

liquid chromatographic method and thin-layer chromatography. However, the radiochemical purity determined by the HPLC method performed according to Ph. Eur. monograph was somewhat higher since acetylated [<sup>18</sup>F]FDG was transformed to [<sup>18</sup>F]FDG under alkaline elution conditions.

#### Analysis of 2-Deoxy-2-Fluoro-D-Glucose (FDG) and 2-Deoxy-2-Chloro-D-Glucose (CIDG)

The developed HILIC-HPLC method was tested for applicability in the determination of chemical purity of [<sup>18</sup>F]FDG. Non-radioactive compounds were identified by a refractive index detector. It was found that using isocratic elution at mobile phase composition of 15:85 (v/v) of 10 mM KH<sub>2</sub>PO<sub>4</sub> buffer (pH7) and acetonitrile FDG and CIDG could be separated with a resolution of 1.8 at 50 µg/mL of excipient concentration (Supplementary information, Fig. S9). Unfortunately, 2-deoxy-2-fluoro-D-glucose and 2-deoxy-2-fluoro-D-mannose (FDM) co-eluted during the HPLC analysis. During the validation of the method a linearity test was performed using the concentration range of 10–100 µg/mL of FDG and CIDG. The obtained regression coefficients (*r*<sup>2</sup>) were in the range of 0.9910 and 0.9986. The repeatability of peak area and retention time of components were evaluated by injection of 6 replicates of the reference

solution at 50 µg/mL concentration of FDG and CIDG. The calculated relative standard deviations (RSD%) were in the range from 0.1 to 3.7%. Additionally, intermediate precision was in the range of 0.1 and 4.3% which was obtained from 6 injections of the reference solution performed on different days. The recovery of FDG and CIDG concentrations were in the range of 90.2 to 112.8%. Limit of quantitation of FDG and CIDG was 40.6 and 44.3 µg/mL, respectively. Robustness was examined at small changes in optimal parameters of flow rate, eluent ratio, pH and ionic strength of mobile phase. The obtained resolutions of FDG and CIDG peaks were in the range of 1.7 and 2.4 (Supplementary information, Table S4). The HPLC analysis of [<sup>18</sup>F]FDG batches revealed that concentrations of FDG and CIDG were below the limit of quantitation.

#### Conclusions

The determination of total radiochemical purity of [<sup>18</sup>F]FDG and [<sup>18</sup>F]FET could be accomplished using HPLC and TLC methods according to the recommendations of Ph. Eur. monographs. In this work, RP-HPLC and HILIC procedures were developed for the separation of radiochemical ingredients of the <sup>18</sup>F-radiopharmaceutical samples. Due to the

difference in polarity of the measured components gradient elution was applied to achieve reasonable peak resolution and measurement time. In the case of [ $^{18}\text{F}$ ]fluoride ions 10 mM potassium dihydrogen phosphate buffer at pH7 is recommended to use as part of the mobile phase to obtain a good peak shape. Additionally, at least 10 mg/mL K[ $^{19}\text{F}$ ]F should be added to the radioactive sample for complete elution of [ $^{18}\text{F}$ ]fluoride ions from the stationary phase. The developed HPLC methods were validated, and good results were obtained for linearity ( $r^2 > 0.998$ ), specificity ( $R_S > 3.7$ ) and repeatability ( $\text{RSD}_\% < 4.3$ ). The application of proposed high-performance liquid chromatography methods could be extended to other  $^{18}\text{F}$ -radiopharmaceuticals for the determination of total radiochemical purity and avoiding additional TLC method.

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**Author Contributions** All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by CAB, CH and IJ. The first draft of the manuscript was written by IJ and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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## Declarations

**Conflict of Interest** The authors have no financial or non-financial interests to disclose.

**Ethical Approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Informed Consent** Informed consent was obtained from all individual participants included in the study.

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