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Improved radiometabolite analysis procedure for positron emission tomography (PET) radioligands using a monolithic column coupled with direct injection micellar/high submicellar liquid chromatography

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ABSTRACT

A high-speed and high-sensitive direct plasma radiometabolite analysis method for positron emission tomography (PET) radioligands has been developed. It was based on micellar/high submicellar liquid chromatography (LC) using a semi-preparative alkyl-bonded silica rod column in conjunction with steep gradient and high flow rate for rapid macromolecule removal from plasma without significant sample preparation and fast/efficient separation of PET radioligands from their radiometabolites. Excellent separation of target PET radioligand from its radiometabolites was achieved within 4 min with a limit of detection of 1 Bq (Bq) level. This method was successfully applied to study the radiometabolism for a wide variety of ^{11}C and ^{18}F labeled radioligands in human or monkey plasma. The improved sensitivity and throughput permitted the analysis of a large number of plasma samples for accurate determination PET radioligands during quantitative PET imaging studies. Micellar/high submicellar LC together with a monolithic column is an attractive alternative method to determine the radiometabolism of PET radioligands in plasma.

1. Introduction

In the positron emission tomography (PET) radiopharmaceuticals field, there is a continuing demand for analytical methods possessing high sample throughput and high sensitivity especially when dealing with plasma samples due to the short half-lives of the positron emitters (*e.g.* ^{11}C: $t_{1/2}=20.4$ min, ^{18}F: $t_{1/2}=109.8$ min) as well as the extensive metabolism and rapid clearance of PET radioligands [1–3]. Liquid chromatography coupled to radioactivity detector (radio-LC) is the most widely accepted technique to determine the relative composition of PET radioligands in plasma [1–4]. However, it often runs into issues with separation due to close retention times for analytes that necessitate a longer analysis time and therefore only a limited number of plasma samples from a PET imaging study can be analyzed (typically ~7 time points). Moreover, certain sample preparation steps such as the protein precipitation and solid-phase extraction to remove proteins from plasma samples are essential prior to the LC analysis to prevent the LC column from clogging and to disrupt protein bound ligands. These are generally achieved manually in off-line mode and therefore, in some cases, become a bottleneck in the analysis process.

Recently, we introduced a micellar/high submicellar liquid chromatographic (MLC/HSC) method to determine PET radioligands in plasma using organic solvents gradients with surfactant-mediated mobile phases [5,6]. This method permits the effective and repetitive analysis of diverse compounds in untreated plasma samples by solubilizing of plasma proteins based on the formation of micellar complex between plasma proteins and anionic surfactants (MLC mode). After the elution of plasma proteins, the condition turns to the HSC to elute radioligands using a high organic solvent content where micelles do not exist. In addition to simplifying the processes the MLC/HSC analysis provides a more accurate determination of PET radioligand.

Monolithic columns, prepared by the sol–gel technology and composed of a single rod of silica-based material, have attracted considerable attention in LC as they allow for achieving good separation faster than the conventional particle-packed columns [7,8]. The unique biporous structure of the monolithic column, where small mesopores (13 nm diameter) provide large surface areas for sufficient separation capacity and larger through pores (2 μm diameter) are relatively biocompatible and provide considerably reduced column back-pressure at high flow rates, allowing high efficiency separation at high flow rates. During the last decade, several papers have been published regarding the use of monolithic columns for high-throughput biological sample analysis [9–14]. Recently, Detroyer et al. established fast MLC methods for quantitative structure retention relationship screening utilizing monolithic

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column and their excellent performance has been demonstrated [15,16].

In this study, we explore the utility of a monolithic silica column in MLC/HSC offering same direct plasma injection as the conventional particle-based column MLC/HSC but with higher sample throughput, higher sensitivity and greater column efficiency for the separation of PET radioligands from their radiometabolites. The performance of the proposed method was demonstrated for the metabolite analysis of a wide array of PET radioligands (Fig. 1) in human and monkey plasma samples taken during PET studies.

2. Experimental

2.1. Chemicals and reagents

Sodium dodecyl sulfate (SDS), phosphoric acid and ammonium hydroxide were obtained from Sigma-Aldrich. Acetonitrile (HPLC grade) was obtained from Fischer Scientific. Authentic samples of PET radioligands were obtained commercially or as gifts and used without further purification. ^{11}C and ^{18}F labeled radiopharmaceuticals were prepared according to the published procedure [17–22].

2.2. Radio-LC system and metabolite analysis conditions

Radio-LC analysis was carried out using a quaternary LC pump (G1311A; Agilent), coupled to a manual injection valve (7725i; Rheodyne) with a 2.0 mL loop. Chromatographic separation was performed on a monolithic alkyl-bonded silica column (Onyx Monolithic Semi-PREP C18, Phenomenex, 100 mm × 10 mm I.D.) using a CH_3CN/50 mM SDS in 10 mM ammonium-phosphate (pH 7) as the mobile phase at a flow rate of 10 mL/min under the following gradient profiles; [^{11}C]flumazenil, [^{11}C]Ro15–4513 and [^{11}C]AZD2184: 5% CH_3CN (0–1.2 min), 5→40% (1.2–2.0 min), 40% (2.0–2.5 min), 5% (2.5–3.5 min), [^{11}C]PBR28: 5% CH_3CN (0–1.2 min), 5→60% (1.2–2.0 min), 60% (2.0–2.5 min), 5% (2.5–3.5 min), [^{11}C]MNPA and [^{18}F]MCL-524: 5% CH_3CN (0–1.2 min), 5→68% (1.2–2.0 min), 68% (2.0–3.0 min), 5% (3.0–4.0 min). The effluent from the column was monitored by an UV absorption detector (G1314D; Agilent) in series with a dual bismuth germanium oxide coincidence radiation detector (S-2493Z; Oyokoken) housed in a shield of 50 mm thick lead. The accumulation time of radiation detector was 3.3 s and the flow cell volume was 500 μL. Data collection and control of the LC system were performed using chromatographic software (EZChrom Elite; Agilent).

2.3. Metabolite analysis of PET radioligands in Human and monkey plasma

The human and monkey PET studies were approved by the Regional Ethics Committee and by the Animal Research Ethical Committee in Stockholm. During PET measurements, whole blood samples were taken from human or monkey and collected in heparin-treated syringes at pre-specified time points after intravenous administration of radioligands. The blood samples were centrifuged at 2000 g for 2–4 min at room temperature to separate plasma. The plasma specimen was then collected and mixed with same volume of a 50 mM SDS solution. The resulting mixture was injected onto the radio-LC system.

3. Results and discussion

Although the potential of MLC for direct injection was demonstrated previously using a conventional silica-based C_{18} column packed with 10 μm particles, poor chromatographic performance in speed, peak capacity and sensitivity were observed even in high submicellar mode [5]. Fast the LC method making use of a short (50–100 mm) small particle-packed column (<3 μm) is an effective alternative, however, a problem associated with increase of back pressure was encountered after injection of untreated plasma into this type of column (XBridge C_{18}, 50 mm × 10 mm I.D., 2.5 μm, 130 Å) even by a pure micellar mobile phase probably due to the clogging of the

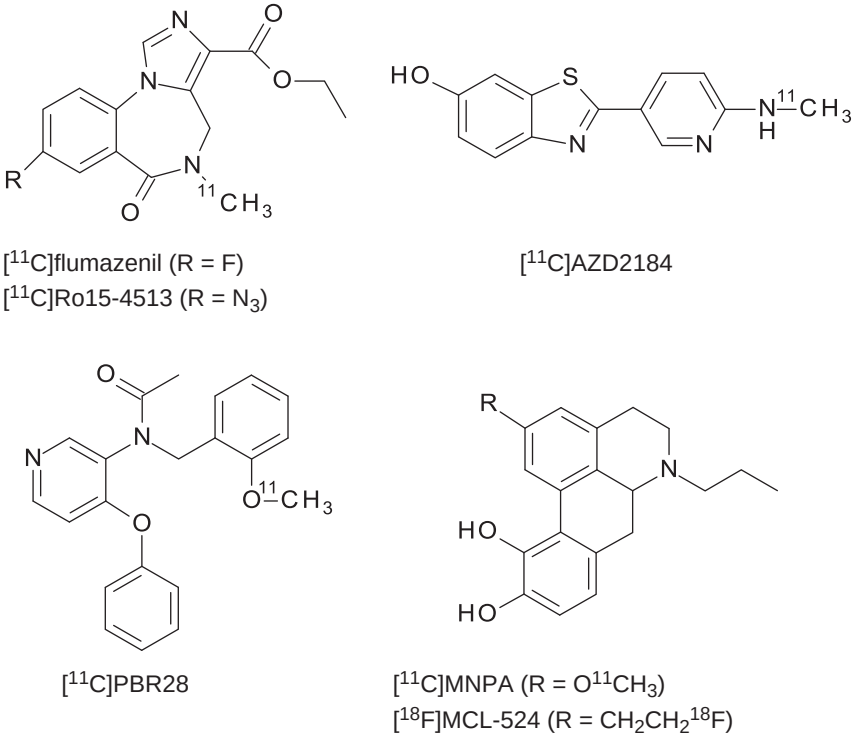

Fig. 1. PET radioligands investigated in this study.

column with hidden particles in the plasma. The use of monolithic columns might overcome this problem and speed up to fast metabolite analysis methods. The dual role of the monolithic column is both to remove matrix macromolecules rapidly and to provide chromatographic efficiency comparable to a conventional microparticulate column for small molecules analysis [9–14].

Efficiency of protein removal was determined by injection of plasma samples (0.1–1.0 mL) spiked with the radiopharmaceuticals using a semi-preparative monolithic column (Onyx Monolithic C18, 100 mm × 10 mm I.D., 130 Å). The use of the monolithic column together with the addition of SDS to the mobile phase reduced the width of the protein band and allowed rapid percolation of endogenous materials through the column. The proteinaceous components were solubilized and flush out completely within 1.2 min using a micellar eluent containing 50 mM SDS, 5% CH_3CN and 10 mM ammonium–phosphate (pH 7) at a flow rate of 10 mL/min without generating excessive back pressure after injection of untreated plasma sample. No breakthrough of radioligands was observed even for strongly protein bound radioligands (*e.g.* plasma protein binding values for [^{11}C]PBR28, [^{11}C]MNPA and [^{18}F]MCL-524: $>90\%$).

Subsequent to complete purification of plasma sample ($t=1.2$ min), the content of acetonitrile in the mobile phase was increased to separate and elute the unmetabolized radioligand and its lipophilic radiometabolites with good peak capacity and shorter retention under high submicellar conditions (CH_3CN: $>25\%$). The elution behaviors of PET radioligands were determined on a monolithic and a small particle column with a high submicellar mobile phase containing 50 mM SDS and 40% CH_3CN at pH 7 (Fig. 2). The efficiency of monolithic column was comparable to 2.5 μm-particule column in high submicellar mode, where theoretical plates (N) of 4000–8000 were obtained for all the six compounds examined. These values were at least 3-times higher than the previous report using a conventional 10 μm-particule column [5]. In contrast to particle packed silica column, the monolithic column generated much flatter Van Deemer plots at high flow rate due to the better mass transfer properties of a monolithic skeleton versus a distinct particles, allowing faster chromatographic separation without a noticeable effect on chromatographic resolution. With monolithic column the maximum column efficiency was maintained up to 10 mL/min (Fig. 2A), while the efficiency was decreased with an increased flow rate (4–7.5 mL/min) by small particle column (Fig. 2B). The flow rate had little influence on the retention factors (k) for PET radioligands in the range of 4–10 mL/min. To shorten the separation time and increase the productivity for repetitive analysis of a large number of samples, the flow rate was adopted to 10 mL/min for monolithic column LC analysis.

Moreover, the monolithic column provided shorter retention times than small particle column. Under equal mobile phase condition, the k values for PET radioligands decreased by 28–43% from the small particle column to monolithic column (Fig. 2).

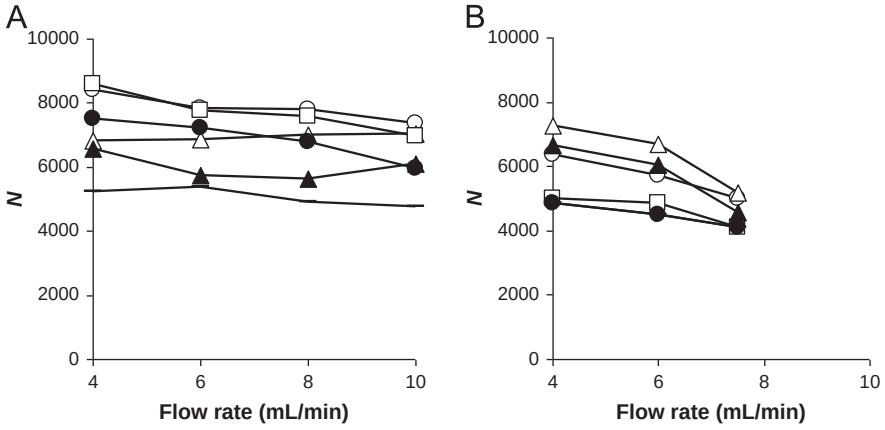

Fig. 2. Effect of the flow rate on theoretical plates (N) for PET radioligands by (A) monolithic column and (B) small particle column MLC. Mobile phase: 40% acetonitrile–50 mM SDS (pH 7), column: (A) Onyx Monolithic C18, 100 mm × 10 mm I.D. and (B) XBridge C_{18}, 50 mm × 10 mm I.D., 2.5 μm, compounds: flumazenil (○), Ro15–4513 (Δ), AZD2184 (□), PBR28 (–), MNPA (●) and MCL-524 (▲).

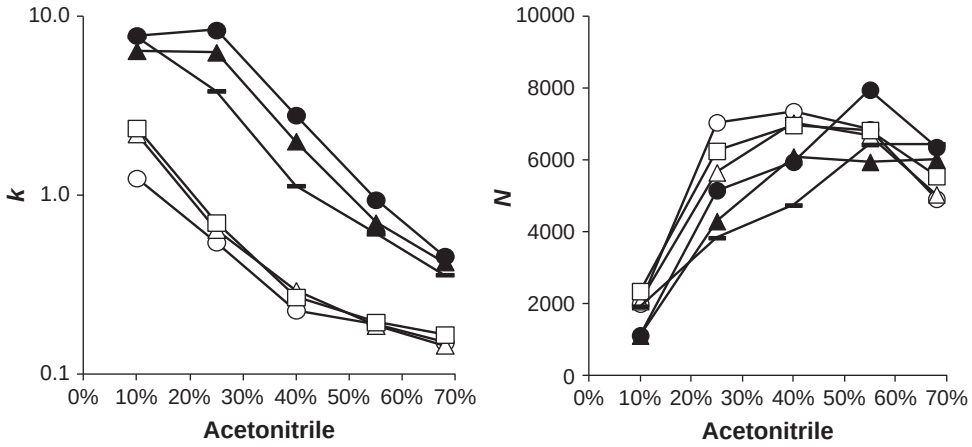

Fig. 3. Dependences of the retention factors (k) and theoretical plates (N) for six PET radioligands with acetonitrile content (%, v/v) in micellar and high submicellar LC modes. Mobile phase: acetonitrile–50 mM SDS (pH 7), Flow rate: 10 mL/min, column: Onyx Monolithic C18, 100 mm × 10 mm I.D., compounds: flumazenil (○), Ro15–4513 (Δ), AZD2184 (□), PBR28 (–), MNPA (●) and MCL-524 (▲).

This low retention of monolithic column may be explained by its high porosity and low density [23,24]. Recently, the use of SDS-acetonitrile mixture in LC has gained much attention due to improved peak efficiency than short-chain alcohols [25–28], however one of the limitations that has restricted the application of this LC is weak elution strength of the mobile phase for eluting retained (moderately to highly hydrophobic) compounds. Thus, it appeared that the use of monolithic column could be useful in reducing retention times. The working flow rates were high, giving faster analysis time without a noticeable efficiency problem.

To optimize the separation conditions for each PET radioligands the chromatographic behaviors of PET radioligands were studied on a monolithic column both in micellar and high submicellar modes (Fig. 3). With conventional particle column MLC/HSC, the separation environments are different depending on the concentration of SDS and acetonitrile present in the mobile phase [25–28]. The monolithic column showed similar retention characteristics as the conventional particle column. In the high submicellar mode hydrophobic interaction is dominant due to decreasing in anionic surfactant coating on the stationary phase, disaggregation of micelle and also decreasing in mobile phase polarity. The k values of PET radioligands decreased significantly with the acetonitrile content in the 25–68% range. In contrast, addition of low concentration of acetonitrile (typically, $<25\%$) increases the micellar size, the CMC and the aggregation

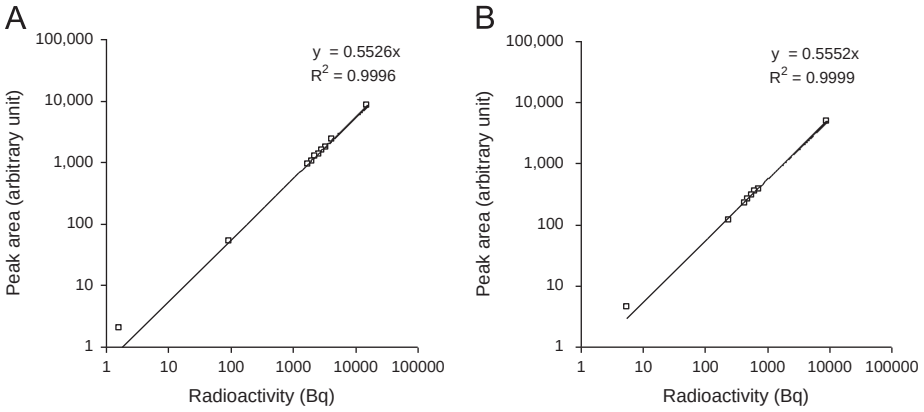

Fig. 4. Calibration curves for (A) [^{11}C]flumazenil and (B) [^{11}C]PBR28 obtained by PET radioligand added to human plasma samples. Plasma sample volume: 0.2–0.5 mL.

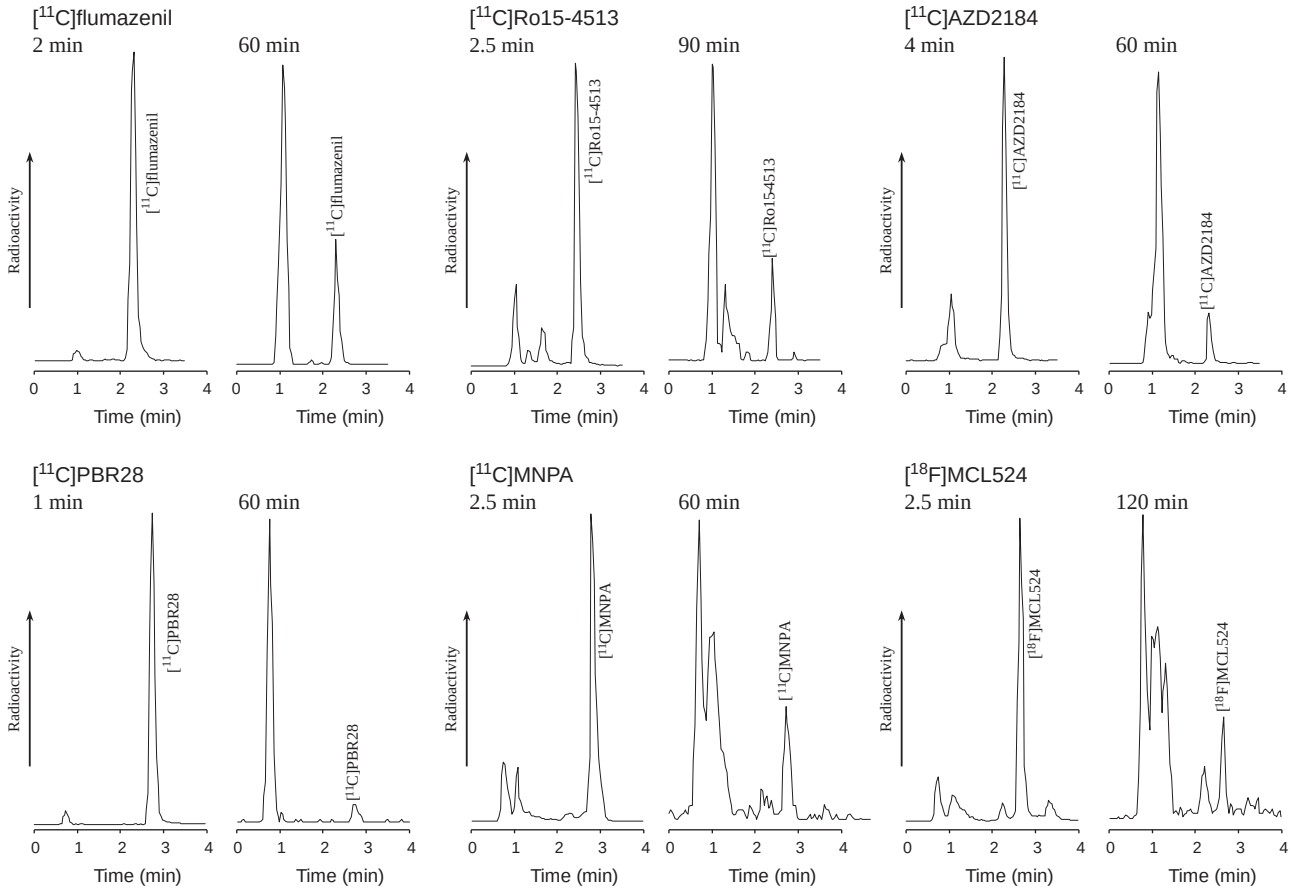

Fig. 5. Radiochromatograms of plasma taken at two different time points after intravenous injection of [^{11}C]flumazenil (human), [^{11}C]Ro15–4513 (monkey), [^{11}C]AZD2184 (human), [^{11}C]PBR28 (human), [^{11}C]MNPA (monkey) and [^{18}F]MCL-524 (monkey) obtained by the monolitic column MLC/HSC method. Plasma sample volume: 0.2–0.5 mL.

number of ionic surfactants and therefore retention times were not significantly affected by acetonitrile in this range. The peak efficiencies were increased with the acetonitrile concentration. The most outstanding enhancements of peak efficiency were observed in the 40–68% acetonitrile range for all compounds with N of 5000–8000 owing to an improvement of poor wetting of stationary phase. For the efficient separation of PET radioligands from their radiometabolites and high-speed analysis of short-lived radioligands, a steep gradient from 5% CH_3CN ($t=1.2$ min) to 40–68% CH_3CN ($t=1.5$ or 2.0 min) was employed.

The optimized chromatographic conditions are shown in experimental section, by which the target PET radioligands were eluted at 2–3 min with a loading capacity of 1 mL plasma. As with the previous study [5], the transition from HSC to MLC was possible with a short equilibration time. Only 1 min was required to re-equilibrate the column with a micellar eluent (5% CH_3CN) after the elution of PET radioligands with a high submicellar mobile phase (40–68% CH_3CN). Total analysis time was below 4 min with low column back-pressure of less than 10 MPa.

Method validation tests were undertaken to evaluate the performance of monolithic MLC/HSC for the determination of PET radioligands in plasma. The relative standard deviations of the retention time and peak area were within 1.0% ($n=6$) and 2.7% ($n=6$), respectively, obtained from repetitive injection of formulation samples at 1000 Bq (Bq) level. The detector exhibited a linear response in the range of at least 10–10,000 Bq with coefficients of determination (r^2) of more than 0.999. The linearity was also evaluated using PET radioligand added to plasma samples. As the examples, Fig. 4 shows human plasma spiked calibration curves for [^{11}C]flumazenil and [^{11}C]PBR28. The linear ranges were at least 10–9000 Bq ($r^2>0.999$). The limits of detection based on the standard deviation (σ) of background (BG) were 1.1–1.3 Bq (BG$+3.3\sigma$), which were at least 2-times better than those by the micellar LC using a regular 10 μm-particle column and a 1-butanol modified SDS mobile phase [5]. The accuracy of the procedure was evaluated using a plasma matrix spiked with known amount of radioactivity (1000 Bq). Sufficient recoveries (97.5–101.4%) were obtained for all compounds tested. The monolithic column showed excellent robustness with nearly 100 injections of plasma (*ca.* total 50 mL plasma) being made onto one column without significant deterioration in performance.

The established monolithic column MLC/HSC method was applied for the determination of PET radioligands in human and monkey plasma to demonstrate its suitability of fast direct analysis. Fig. 5 shows typical radiochromatograms obtained by plasma taken at two different time points after intravenous injection of six radioligands. Complete separations of target PET radioligands from their radiometabolites were achieved within 4 min without significant sample preparation of plasma. Up to 19 plasma samples could be analyzed in one 60 min PET study of ^{11}C-labeled radioligands, thus overcoming the limitation of the conventional radio-LC in only allowing metabolite analysis for a limited number of plasma used to derive a metabolite corrected input function. The high sensitivity provided by the present method enabled metabolite analysis with lower sample volume (0.2–0.5 mL plasma for monolithic column MLC/HSC versus ~1.5 mL plasma for conventional LC) and facilitated minimizing the volume of withdraw blood samples from subjects.

4. Conclusion

The direct PET metabolite analysis of plasma samples has been demonstrated by monolithic silica column MLC/HSC. The use of the monolithic column resulted in increased sensitivity, better resolution and shorter analysis time compared to a conventional particle based column. It speeded up the process not only by reducing tedious sample preparation steps but also by fast chromatographic separation and therefore significantly facilitated the high-throughput metabolite analysis of a large number of plasma samples for improved determination of PET radioligands during quantitative PET imaging studies.

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