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Research Article

Use of LC–MS–MS for the rapid, specific and sensitive quality control measurement of carrier in a PET radioligand: [^{18}F]FECNT

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Summary

In the production of radioligands for imaging low concentrations of target proteins (e.g. receptors or transporters) in human subjects with positron emission tomography, control of specific radioactivity is necessary for efficacy and safety. Such quality control requires a fast method to be available for measuring carrier (non-radioactive ligand) in each batch of radioligand, preceding its release for administration. Measurement is usually achieved with HPLC equipped with an ultraviolet (UV) absorbance detector. However, this method is not easily applicable to radioligands that have low UV extinction coefficients and are produced at high specific radioactivity, such as [^{18}F] (2β-carbomethoxy-3β-(4-chlorophenyl)-8-(2-fluoroethyl)-nortropane; [^{18}F]FECNT). Here we describe a fast, specific and sensitive LC–MS–MS method for measuring carrier in [^{18}F]FECNT preparations. Small samples of formulated [^{18}F]FECNT plus an added internal standard (2β-carbomethoxy-3β-(4-chlorophenyl)-8-(*n*-propyl)nortropane; INTSTD) are rapidly eluted from a short reverse phase HPLC column into an MS probe. Following electrospray ionization, the molecular ions ([MH]$^+$) of FECNT ($m/z = 326$) and INTSTD ($m/z = 322$) are isolated and energized for collision-induced dissociation. The product ions from FECNT ($m/z = 294$) and INTSTD ($m/z = 290$) are monitored selectively. The calibration curve for MS response is linear for FECNT concentrations in the range 2–20 pg/μl and suitable for reproducibly (RSD 5%) and rapidly (< 3 min) measuring low concentrations of carrier in [^{18}F]FECNT preparations. Copyright © 2005 John Wiley & Sons, Ltd.

Key Words: LC–MS–MS; carrier; [^{18}F]FECNT; radioligand; PET

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Introduction

Positron emission tomography (PET) has become an important technique for imaging specific biochemical targets *in vivo* as part of clinical research[1–3] or drug development.[4,5] The technique depends on the use of biochemically specific radiopharmaceuticals (radiotracers/radioligands), which are usually labeled with short-lived carbon-11 ($t_{1/2} = 20.4$ min) or fluorine-18 ($t_{1/2} = 109.7$ min). Radioligands are produced for the PET imaging of low concentrations of target proteins (e.g. receptors, transporters or enzymes) in the organs of animals or human subjects. Control of the amount of carrier (non-radioactive ligand) present with the radioligand and of the specific radioactivity (radioactivity per unit mass of carrier) of the radioligand are necessary for both safety and efficacy.[6,7] Such quality control requires a fast and sensitive method to be available for the measurement of carrier in each batch of the radioligand before its release for administration. This is usually achieved with HPLC equipped with an ultraviolet (UV) absorbance detector.[8] However, this method is not easily applicable to radioligands that have low UV extinction coefficients or that have carrier present at very low concentration. Moreover, this technique is not necessarily specific for detection of the carrier since there is a risk that occasionally a low level of a known or unknown impurity may overlap with the carrier fraction eluted in HPLC. [^{18}F](2β-Carbomethoxy-3β-(4-chlorophenyl)-8-(2-fluoroethyl)nortropane; [^{18}F]FECNT), a dopamine transporter radioligand,[9] is an example of a radioligand having a low UV extinction coefficient, rendering the measurement of carrier difficult by HPLC with absorbance detection. Here we describe an alternative fast, specific and sensitive LC–MS–MS[10] method for measuring carrier FECNT (Figure 1(a)) in preparations of [^{18}F]FECNT for human administration.

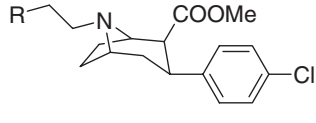

Figure 1. Chemical structures of (a) FECNT (R = F) and (b) INTSTD (R = Me)

Results and discussion

Selection of an internal standard

An LC–MS method of quantification ordinarily involves use of an internal standard such as a stable isotope or a structural analog having a molecular mass or chromatographic retention time different from that of the compound being quantified. A structural analog of FECNT, 2β-carbomethoxy-3β-

(4-chlorophenyl)-8-(*n*-propyl)nortropane (INTSTD; Figure 1(b)), which differs in molecular weight from FECNT by only 4 amu, was readily prepared by treating 2β-carbomethoxy-3-β-(4-chlorophenyl)-8-nortropane with 3-iodopropane. The chromatographic mobility and electrospray-MS and MS–MS fragmentation characteristics of FECNT and INTSTD were found to be similar. The most abundant fragment ion from each compound was chosen in selected reaction monitoring and generation of the ion chromatograms for quantification.

Electrospray-MS and collision-induced dissociation of FECNT and INTSTD

Electrospray-MS analysis of FECNT generated the molecular ion ($[MH]^+$; $m/z = 326$) paired with $m/z = 328$ in expected relative abundance for the presence of ^{37}Cl isotope. Using an appropriate isolation (mass) width, the parent ion ($m/z = 326$) was isolated and energized, and the resulting fragment ions were scanned. The parent ion spectrum of FECNT is shown in Figure 2(a) (inset) and the product ion spectrum (MS–MS), resulting from collision-induced dissociation of the $m/z = 326$ ion, shown in Figure 2(b). The internal standard, INTSTD, was similarly analyzed and the parent ion spectrum is shown Figure 3(a) (inset) and the product ion spectrum shown in Figure 3(b). Electrospray ionization of INTSTD generated the molecular ion ($m/z = 322$) accompanied by $m/z = 324$ ion for the presence of ^{37}Cl isotope. Isolation and

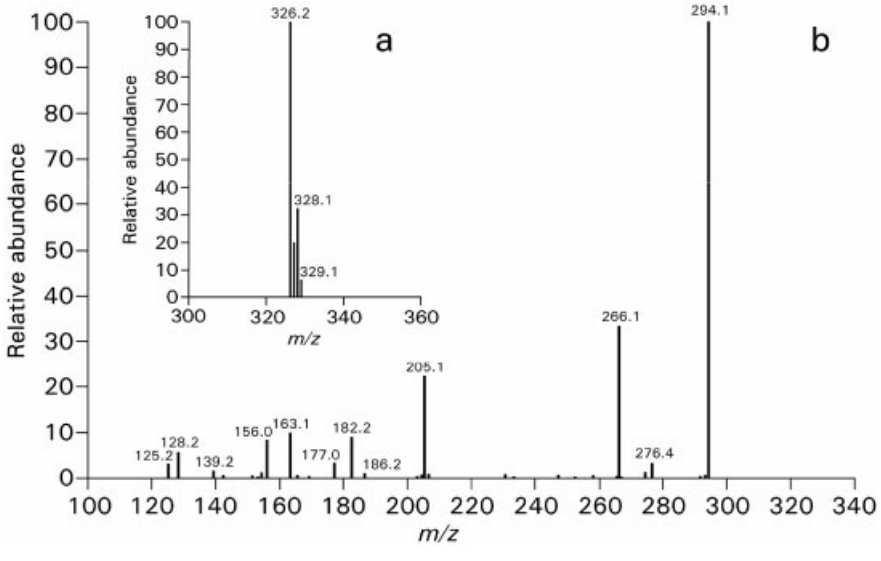

Figure 2. (a) Parent-ion ($[MH]^+$) mass spectrum of FECNT showing the $m/z = 326$ ion generated by electrospray-ionization and (b) product-ion spectrum resulting from collision-induced dissociation of $m/z = 326$ ion

energization of the parent ion ($m/z = 322$), generated characteristic product ions analogous to those obtained for FECNT.

In the product ion spectrum of FECNT (Figure 2(b)), the most abundant fragment ion ($m/z = 294$) is likely to be generated by the loss of neutral MeOH from the protonated FECNT, as shown in Figure 4(a). In a similar process, the parent ion ($m/z = 326$) upon energization can eliminate AcOH, and the product ion ($m/z = 266$) can account for the loss of this neutral species. The structures for the remaining less abundant fragment ions are not readily assignable. The fragmentation pattern for cocaine, a tropane analog, has been well documented.[11,12] However, FECNT does not fragment analogously because of the 4–chlorophenyl substituent on the tropane ring.

The product ion spectrum for INTSTD is similar to that for FECNT. The major fragment ion ($m/z = 290$) (Figure 3(b)) is likely to be formed by the loss

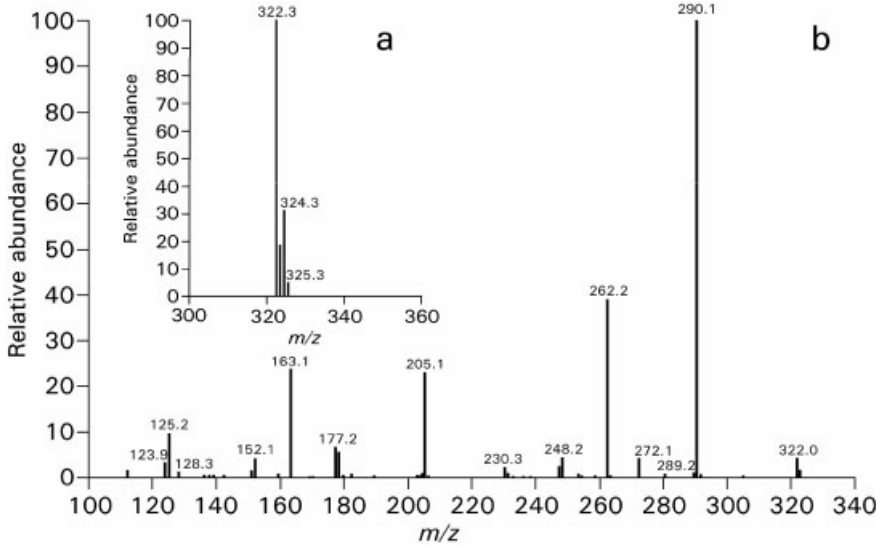

Figure 3. (a) Parent-ion ($[MH]^+$) mass spectrum of INTSTD showing the $m/z = 322$ ion generated by electrospray-ionization and (b) product-ion spectrum resulting from collision-induced dissociation of $m/z = 322$ ion

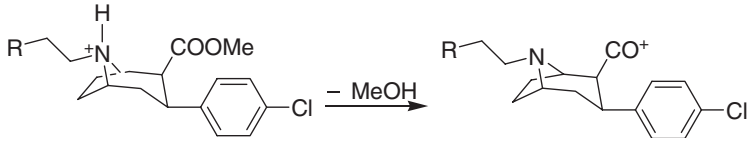

Figure 4. Proposed fragmentation pathway for the protonated FECNT ((a) R = F) and INTSTD ((a) R = Me) and the structures of respective product ions $m/z = 294$ ((a) R = F) and 290 ((b) R = Me)

of MeOH from the protonated INTSTD (Figure 4(b)). The selected reaction monitoring in the FECNT quantification involved acquisition of the most abundant fragment ions of FECNT ($m/z = 294$) and of INTSTD ($m/z = 290$), as described.

Liquid chromatography

The duration of an LC–MS–MS run is mainly determined by the time required for autosampling and injection plus the HPLC retention time of the analyte. The time utilized by the autosampler is usually fixed. However, the chromatographic retention time is determined by the column length, stationary-phase chemistry and composition of the mobile phase. Once the analyte elutes from the column, negligible time is spent on completion of the MS analysis involving electrospray ionization and subsequent passage (through ion optics) and deposition of ions into the mass analyzer. A narrow-bore, short reverse phase (C-18) column was chosen to enable a rapid elution of FECNT along with INTSTD. The HPLC mobile phase, a mixture of water and methanol, was pumped isocratically, rather than in gradient mode, in order to reduce the time required to equilibrate the column and to maintain a steady solvent composition for electrospray ionization. The retention time of FECNT on the column was one minute and the INTSTD almost co-eluted with FECNT under these chromatographic conditions. Thus, both compounds undergo chromatographic separation, ionization and detection in almost identical time and space domains. The analytical process involved here is similar to that of a MS quantification using a stable isotope as internal standard. The chromatographic peaks, generated by monitoring $m/z = 294$ and 290 ions, were symmetrical for both FECNT and the INTSTD (Figure 5(a) and (b)).

Calibration curve

Following acquisition of a data set, the software plotted the calibration curve as FECNT concentrations (*X*-axis) *versus* FECNT to INTSTD peak area ratios (*Y*-axis). The calibration curve was linear for FECNT concentrations between 2 and 20 pg/μl; the corresponding absolute amounts of FECNT are in the range 10–100 pg, since 5 μl of each standard were injected into the LC–MS instrument. Thus, about 31 fmol (injected) of FECNT was the lower limit of quantification with this curve. A representative FECNT calibration curve, exhibiting slope 0.246, intercept −0.218, and correlation (r^2) 0.996, is shown in Figure 6. The calibration curve encompasses the concentrations of carrier present in microliter quantities of twenty-fold diluted [^{18}F]FECNT preparations. The calibration curve was reproducible and its accuracy

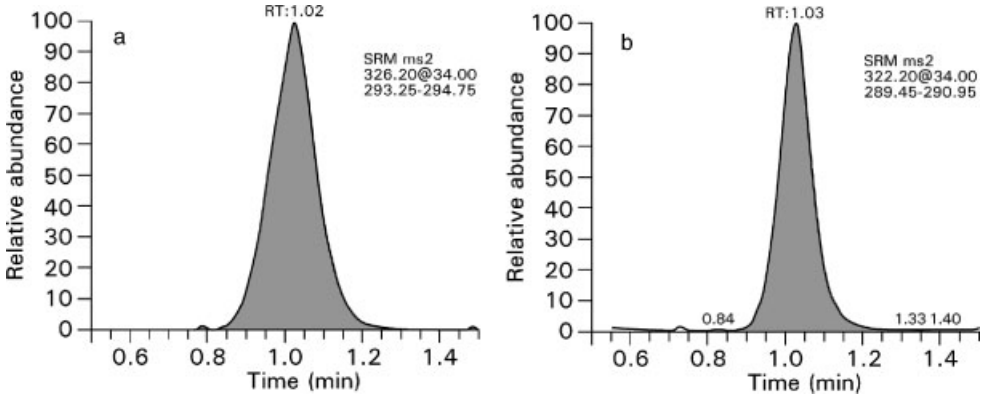

Figure 5. (a) Selected reaction monitoring of the ion-transition $m/z = 326 \rightarrow 294$ and the resulting ion chromatogram for FECNT in a ^{18}F-labeled preparation and (b) of the ion-transition $m/z = 322 \rightarrow 290$ and the ion chromatogram for INTSTD

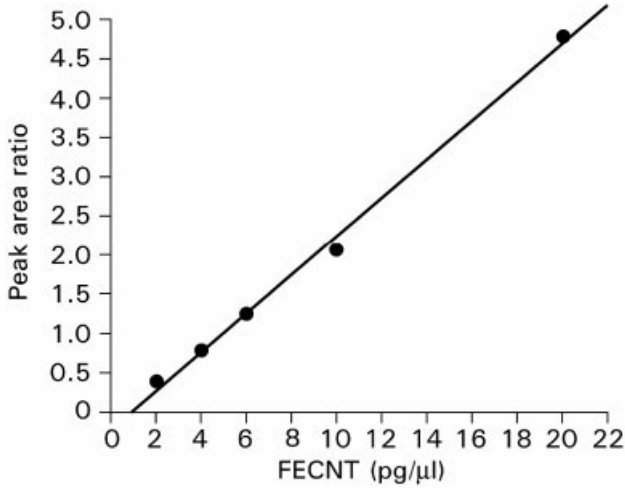

Figure 6. A representative calibration curve for FECNT: $y = 0.246x - 0.218$, $r^2 = 0.996$

for reporting the concentrations was satisfactory. For example, curves generated on different days ($n = 8$) reported a concentration of 3.99 ± 0.31 (mean $\pm$ SD) pg/µl for a specified concentration of 4.0 pg/µl of FECNT standard.

Reproducibility of the quantification

Precision of the measurement, as defined by same-day and between-day variations, was evaluated by performing replicate analysis of the carrier

Table 1. Same-day and between-day precision data for the quantification of FECNT carrier in the [^{18}F]FECNT preparations

Precision type	*n*	Mean ± SD concentration (ng/ml)	RSD[a] (%)
Same-day	6	246 ± 12.2	4.9
Between-day	5	194 ± 18.4	9.5

[a] Relative standard deviation (%) = (SD/mean) × 100.

in a [^{18}F]FECNT preparation. Six replicate samples were drawn from an ^{18}F-labeled preparation and the amounts of carrier in each were quantified to determine same-day variations. The concentrations (mean) and standard deviations (SD) are shown in Table 1. The RSD value of 4.9% is indicative of minimal sample-to-sample variation and good reproducibility of the LC–MS–MS quantification. Another FECNT preparation was analyzed on five different days, each time using a new calibration curve to determine the concentration. The between-day data (Table 1) for FECNT measurements also confirms the reproducibility of the method.

Quantification of FECNT in the ^{18}F-labeled preparations

The LC–MS–MS method provides the concentration of the carrier and therefore allows the specific radioactivity to be determined in a small volume of formulated [^{18}F]FECNT preparation. The analysis involves twenty-fold dilution of the sample and injection of 1% of the diluted sample into the LC–MS to acquire the data. As a negligible amount of the radioligand enters the LC–MS system, no specialized containment of the radioactivity is necessary. With the prior acquisition of calibration curve data, the concentration of the carrier is obtained in about 2 min. Figure 5 shows ion chromatograms generated by the MS–MS acquisition of $m/z = 294$ ion for FECNT and $m/z = 290$ ion for INTSTD for a representative [^{18}F]FECNT preparation. Results of the LC–MS–MS analysis of the carrier in five different [^{18}F]FECNT preparations and the calculated specific radioactivities are shown in Table 2. In these preparations, specific radioactivity ranged between 107 and 255 GBq/μmol (2.9 and 6.9 Ci/μmol) with a mean ± SD value of 153 ± 60 GBq/μmol (4.1 ± 1.6 Ci/μmol).

During radiochemical syntheses of [^{18}F]FECNT, samples were also injected into HPLC (UV absorbance detection) and peak areas for the carrier were measured. The concentrations of the carrier FECNT were obtained from a calibration curve for FECNT with no internal standard added. Although no attempt was made to compare and validate the results, the specific radioactivity obtained by HPLC measurements (159 ± 64 GBq/μmol; mean ± SD) was found to be very similar to that obtained by measuring the carrier by the LC–MS–MS method.

Table 2. Concentrations of the carrier measured by LC–MS–MS and the calculated specific radioactivity for [^{18}F]FECNT preparations

[^{18}F]FECNT preparation	Amount of FECNT in 25 µl sample (ng)	FECNT concentration (nmol/ml)	Radioactivity (mCi/ml) or [MBq/µmol]	Specific radioactivity (Ci/µmol) or [GBq/µmol]
A	4.18	0.514	2.1 [77.7]	4.1 [152]
B	7.03	0.865	2.5 [92.5]	2.9 [107]
C	3.02	0.372	1.4 [51.8]	3.8 [141]
D	11.58	1.425	4.2 [155]	3.0 [111]
E	6.14	0.756	5.2 [192]	6.9 [255]

Experimental

Chromatographic grade solvents and acetic acid were purchased from EM Science (Gibbstown, NJ) and all other reagents were from Sigma-Aldrich (Milwaukee, WI). 2β-Carbomethoxy-3β-(4-chlorophenyl)-8-nortropane (CNT), the precursor for the synthesis of INTSTD and [^{18}F]FECNT, and FECNT were synthesized and supplied by Dr Mark M. Goodman (Emory University, Atlanta, GA). The structural identity and chemical purity (>99%) of these compounds were ascertained by NMR, MS and HPLC analyses. Proton and ^{13}C NMR spectra were recorded in $CDCl_3$ containing 0.03% TMS using an Avance 400 MHz spectrometer (Bruker; Billerica, MA). All chemical shifts (δ) are reported in ppm. Melting point was determined in an open capillary using Electrothermal Mel-Temp® apparatus. A Beckman-Coulter (Fullerton, CA) HPLC equipped with Waters (Milford, MA) column was used for purifying [^{18}F]FECNT and the intermediates.

Synthesis of 2β-carbomethoxy-3β-(4-chlorophenyl)-8-(n-propyl)-nortropane (INTSTD)

Triethylamine (45 µl; 0.32 mmol) was added to a solution containing CNT (15 mg; 54 µmol), *N*,*N*-dimethylformamide (250 µl) and iodopropane (32 µl; 0.32 mmol). The reaction mixture was stirred overnight at room temperature and subsequently diluted with water (6 ml) and extracted with dichloromethane (2 × 3 ml). The organic fractions were combined, dried over anhydrous magnesium sulfate and evaporated with a Kugel Rohr apparatus to afford pure product (14.2 mg; 82.3%) as pale yellow crystals, mp 66–68°C; ^{1}H NMR (400 MHz, $CDCl_3$/TMS) δ 0.87 (t, J = 7.3 Hz, 3H), 1.38 (m, 2H), 1.65 (m, 3H), 2.05 (m, 2H), 2.20 (m, 2H), 2.56 (dt, J = 2.6, 12.4 Hz, 1H), 2.88 (m, 1H), 2.97 (m, 1H), 3.39 (t, J = 2.9 Hz, 1H), 3.50 (s, 3H), 3.70 (t, J = 3.3 Hz, 1H), 7.21 (dd, J = 2.0, 8.7 Hz, 4H); ^{13}C NMR (400 MHz, $CDCl_3$/

TMS) δ 11.96, 22.41, 26.00, 26.34, 34.06, 34.28, 51,23, 53.01, 55.79, 61.93, 62.73, 128.16, 128.96, 131.56, 142.12, 172.16.

Radiosynthesis

[^{18}F]FECNT was prepared with the method[9] described previously, modified so that the intermediate [^{18}F]1-fluoro-2-tosyloxyethane was purified by HPLC (acetonitrile-water gradient; RP_{18} Xterra column 7.8 mm × 300 mm; Waters) and then isolated in acetonitrile *via* solid phase extraction. The ^{18}F-labeled alkylating agent was then reacted with CNT (0.75 mg) by concentrating the acetonitrile in an open, heated vessel (110°C; 10 min). A helium sweep gas (10 ml/min) was used to facilitate removal of the acetonitrile. HPLC on the Xterra column, described above, eluted with an acetonitrile-10 mM ammonium hydroxide (pH = 10) gradient afforded [^{18}F]FECNT of high chemical and radiochemical purity. Specific radioactivity was determined at the end of synthesis by HPLC on a Luna C-18 column (10 μm; 4.6 × 250 mm; Phenomemex) eluted with acetonitrile-10 mM ammonium formate (*aq.*) (1: 1 v/v) at 2 ml/min. Compounds were detected by absorbance at 229 nm. Carrier FECNT was determined by use of a calibration curve for FECNT (without an internal standard). HPLC measurement of the carrier in ten different [^{18}F]FECNT preparations gave specific radioactivity values in the range 72–39 GBq/μmol (1.94–6.46 Ci/μmol) with a mean ± SD value of 159 ± 64 GBq/μmol (4.29 ± 1.74 Ci/μmol).

Instrumentation set up

The LC–MS and MS–MS analyses were performed on an LCQ Deca model LC–MS (Thermo Electron Corporation; San Jose, CA). This LC–MS system included a Surveyor LC (or HPLC) and Surveyor autosampler. The HPLC mobile phases were water–methanol–acetic acid (90: 10: 0.5 by vol.) (mobile phase A) and methanol–AcOH (100: 0.5 v/v) (mobile phase B). The HPLC pump was set to deliver an isocratic mixture of 65% A and 35% B at 150 μl/min. The chromatography was performed on a reverse-phase column (Luna C18; 50 (l) × 2 (i.d.) mm; 5 μm; Phenomenex Torrance, CA). The whole output of the HPLC column was introduced into the MS probe for electrospray ionization. The source voltage for electrospray was 4.5 kV and the capillary voltage was 16 V. The capillary temperature was 260°C. For nebulization, the sheath gas (nitrogen) flow rate was set at 44 units. The MS was set up in the MS–MS mode for acquiring and dissociating $m/z = 326$ and 322 ions and for selected reaction monitoring of $m/z = 294$ and 290 product ions generated from FECNT and INTSTD, respectively. The MS acquisition time was 1.5 min.

Collision-induced dissociation (MS–MS analysis) of FECNT

The MS was tuned by infusing a solution of FECNT *via* the HPLC eluate line into the electrospray unit and maximizing transmission of ions into the MS analyzer and detector. The solution of FECNT (1 ng/μl in mobile phase) was infused at 5 μl/min into the LC flow (flow rate 150 μl/min) line using a T-joint and the molecular ion ($[MH]^+$; $m/z = 326$) was observed in the tune window. The voltages for various components were automatically optimized for the transmission of the $m/z = 326$ ion across the heated capillary and ion optics and deposition into the MS analyzer (ion trap). The tune file was saved and incorporated into the instrument setup for acquisition.

After the mass spectrometer had been tuned for the $m/z = 326$ ion, MS–MS analysis was performed on FECNT to identify the most abundant product ion. The effect of isolation width (in m/z units) on the intensity of the parent ion ($m/z = 326$), was examined and a value of $m/z = 1.5$ was found to be optimal. The isolated parent ion was energized at a collision energy set at 30% level and the daughter (product) ion spectrum was observed in the tune window. The product ion ($m/z = 294$) was identified as the most abundant ion in the ion chromatogram and the collision energy for this ion was optimized automatically. The collision energy at a 34% level was found to be optimal for the transition of the $m/z = 326$ ion to the $m/z = 294$ ion. The same isolation width and collision energy were used for the isolation and dissociation of the parent ion ($m/z = 322$) of the internal standard, INTSTD. The most abundant product ion ($m/z = 290$) was selected for monitoring the internal standard.

Processing and reporting of LC–MS–MS data

A processing method was set up to construct the ion chromatograms, integrate the peak areas, plot FECNT concentrations *versus* peak area ratios of FECNT to the internal standard and to calculate and report the concentration of FECNT in an unknown sample. The specific ions, $m/z = 294$ of FECNT and $m/z = 290$ of INTSTD, were extracted from the total ion chromatogram and appropriate smoothing points and area and peak noise factors were applied for a reliable peak integration of the components. A linear calibration curve was set up in the method for plotting the FECNT concentrations, ranging between 2 and 20 pg/μl, and the respective peak area ratios. The processing method reported the calibration curve data as well as the concentration of carrier FECNT in an unknown sample at the end of analysis of a sample set.

Calibration curve solutions

A stock solution containing FECNT (10 ng/μl) and another containing INTSTD (1 ng/μl) were prepared in acetonitrile and stored at −20°C. Before

analysis, the stock solution of INTSTD was brought to room temperature and an aliquot was diluted with acetonitrile to obtain a 100 pg/μl working solution. The stock solution (50 μl) of FECNT was diluted with water (to 10 ml) to obtain a 50 pg/μl working solution. Calibration curve solutions were prepared by pipetting 20, 40, 60, 100 or 200 μl aliquots of standard FECNT solution (50 pg/μl) into an autosampler vial, adding 50 μl of INTSTD solution (100 pg/μl) to each and finally diluting the content of each vial with water to a final volume of 500 μl. Thus, the final concentrations of FECNT in these vials were 2, 4, 6, 10 and 20 pg/μl. Five microliters of solution from each vial were injected into the LC–MS to acquire the calibration curve data.

Sample preparation

Carrier in [^{18}F]FECNT preparations was quantified immediately after production or after substantial radioactivity decay. Twenty five microliters of each sample were transferred to an autosampler vial, mixed with 50 μl of INTSTD solution and then diluted with water to a final volume of 500 μl, as for the calibration curve solutions. The autosampler injected 5 μl of this sample solution into the LC–MS. For same-day variability data, replicate ($n = 6$) samples were similarly prepared from a single [^{18}F]FECNT preparation and analyzed. Additionally, six replicate samples (25 μl each) from another batch of [^{18}F]FECNT were stored at -20°C and each sample was analyzed on a different day.

Conclusions

A rapid, specific and sensitive LC–MS–MS method was developed for measuring FECNT carrier in [^{18}F]FECNT preparations. This method required only a very small amount of formulated [^{18}F]FECNT preparation as analyte. During the analysis, only a minuscule amount of radioactivity (< 2 μCi) entered the MS system and radioactivity containment was not required. Quantification of carrier was achievable in less than 3 min, preceding release of the radioligand for human administration. This type of LC–MS–MS procedure might be used more widely for the quantification of radiopharmaceutical carriers that are otherwise difficult to detect with high specificity, sensitivity and accuracy.

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