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Labeling of human C-peptide by conjugation with *N*-succinimidyl-4-[^{18}F]fluorobenzoate

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Summary

We have labeled proinsulin connecting peptide (C-peptide) with fluorine-18 ($t_{1/2} = 109.7$ min) in order to perform *in vivo* biodistribution and pharmacokinetic studies with position emission tomography (PET). This study reports the optimization of the conjugation labeling in the N-terminal with *N*-succinimidyl-4-[^{18}F]fluorobenzoate ([^{18}F]SFB). In preparative runs *N*-4-[^{18}F]fluorobenzoyl-C-peptide ([^{18}F]FB-C-peptide) was produced in 8–12% decay-corrected yields, counted from resolubilized [^{18}F]F^-, in less than 5 h. The specific radioactivity of [^{18}F]FB-C-peptide, determined using ELISA for one of the preparations, was around 70 GBq/μmol at end of synthesis. Copyright © 2001 John Wiley & Sons, Ltd.

Key Words: fluorine-18; protein labeling; connecting peptide; diabetes; PET

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Introduction

Insulin is synthesized in the pancreatic beta cells from a larger precursor molecule, proinsulin. The latter is shown in Figure 1 and consists of an A-chain and a B-chain, linked by a 31 amino acid connecting peptide (C-peptide). C-peptide is postulated to facilitate alignment of the A and B chains for the formation of disulfide bonds between them.[1] C-peptide is subsequently cleaved from proinsulin, equivalent amounts of C-peptide and insulin are stored in secretory granules and eventually released to the circulation following glucose stimulation. C-peptides has been considered to possess little, if any, biological activity. However, a series of recent reports has demonstrated that C-peptide administration to patients with insulin-dependent diabetes mellitus (IDDM) results in increased blood flow to skeletal muscle and skin, improved renal function and amelioration of diabetic neuropathy (see Reference 2 for a review of C-peptide effects).

It is known that C-peptide is both metabolized and excreted by the kidneys.[3,4] Other than that, little is known of the pharmacokinetics of

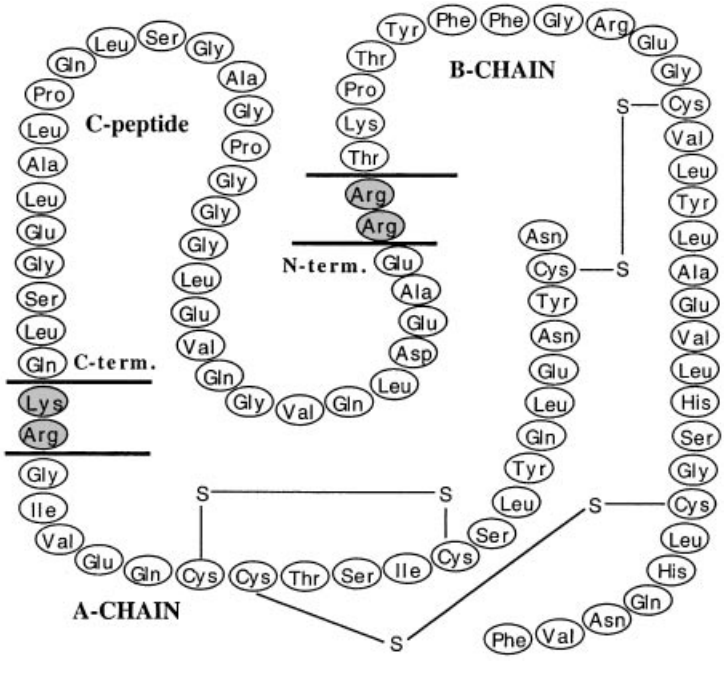

Figure 1. Structure and amino acid sequence of human proinsulin, with A- and B-chains of insulin and C-peptide

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C-peptide. We have developed a procedure for labeling human C-peptide with fluorine-18 (previously described briefly in Fredriksson *et al.*[5]) with the aim to non-invasively evaluate the peptide biodistribution and pharmacokinetics using positron emission tomography (PET).

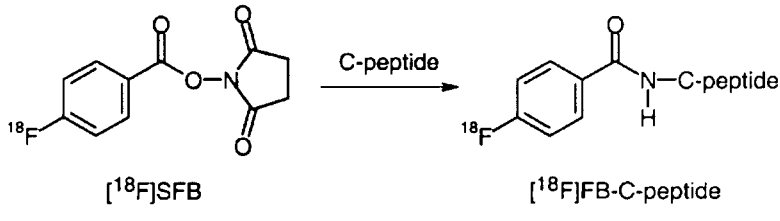
A number of methods for labeling proteins and peptides have been previously reported (reviewed in Reference 6). There are no aromatic amino acids in C-peptide (Figure 1), which excludes the frequently used route of direct oxidative halogenation. There are a few ^{11}C-labeled reagents suitable for labeling peptides under mild conditions (for example, Reference 7). However, C-peptide contains several amino acids reactive toward those reagents and a site-directed labeling would be difficult to achieve.

In vitro studies have revealed that C-peptide stimulates Na^+K^+ATPase and that the major part of the stimulating activity is inherent to the five amino acid sequence of the C-terminal whereas the N-terminal has essentially no activating effect.[2] We have therefore chosen to label the peptide with a prosthetic group attached to the N-terminal. *N*-Succinimidyl-4-[^{18}F]fluorobenzoate ([^{18}F]SFB) was chosen (Scheme 1) based on reports in the literature[8–11] that it could be used under mild conditions of pH and temperature for peptide and protein labeling of primary amine groups from, for example, lysine or the N-terminal.

Experimental

General

All reagents, unless otherwise specified, were of analytical grade and commercially available. Dimethylsulfoxide (DMSO) was distilled from



Scheme 1. Labeling of C-peptide by conjugation of [^{18}F]SFB to the N-terminal, giving *N*-4-[^{18}F]fluorobenzoyl-C-peptide ([^{18}F]FB-C-peptide)

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barium oxide, dispensed under N_2 into vials containing activated molecular sieves (4 Å) and stored at 4°C. Anhydrous acetonitrile (MeCN) from a newly opened flask was stored over activated molecular sieves (4 Å) at room temperature (r.t.). All other solvents were used without further purification. 4,7,13,16,21,24-Hexaoxa-1,10-diazabicyclo-[8.8.8]hexacosan (Kryptofix® 2.2.2) and potassium carbonate (K_2CO_3), as well as the boric acid and borax used for the borate buffer, were obtained from Merck; 4-(dimethylamino)benzonitrile, methyl trifluoromethane-sulfonate and *N*, *N*′-disuccinimidyl carbonate from Aldrich; tetrabutylammonium hydroxide in MeOH (25%), 4-fluorobenzonitrile and *N*-hydroxysuccinimide from Fluka and *N*, *N*′-dicyclohexylcarbodi-imide (DCC) from Janssen Chimica. The human C-peptide was synthetically produced via a Merrifield solid phase synthesis (PolyPeptides, Wolfenbüttel, Germany).

Melting points are reported uncorrected and were determined with an Electrothermal IA9000 series digital melting point apparatus. Radio-TLC was performed using silica gel 60 F_{254} plates (Merck), a Bioscan system 200 and Winscan software, version 2.2.

Radioanalysis by HPLC was performed using two Shimadzu LC-10AD pumps, A Shimadzu SPD-6A spectrophotometric detector, a Beckman model 170 radioisotope detector and a Shimadzu C-R4AX integrator. A Nucleosil C8 column (Macherey-Nagel, 4.6×200 mm, 7 μm) was used with mobile phase of A (MeCN containing 0.085 vol% trifluoroacetic acid (TFA)) and B (distilled water (dH_2O) containing 0.1 vol% TFA). The mobile phase composition was changed by increasing A in a linear gradient from 20 to 40% during 15 min, plateaued at 40% for 3 min and each analysis was ended by washing with 80% A. The UV-wavelength was 220 nm for the individual radioanalyses and 236 nm for the [^{18}F]SFB specific radioactivity determinations. The flow rate was 2 ml/min.

For HPLC purification of [^{18}F]SFB, a Shimadzu LC-6A pump, a SpectroMonitor II UV (254 nm) and a GM-tube (Studsvik mini-monitor, type 5.10) were used. A μPorasil column (Waters, 7.8×300 mm, 10 μm) was eluted with *n*-hexane: ethyl acetate: TFA = 88: 12: 0.12 at 4.0 ml/min.

The labeled peptide was purified with HPLC using a Nucleosil C8 column (Phenomenex®, 10.0×250 mm, 5 μm) and a linear gradient of A and B (as above) with A increasing from 20–80% over 60 min, with a flow rate of 4.0 ml/min. The HPLC system consisted of two Shimadzu

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LC-10AD pumps, a Gilson 115 UV absorbance detector at 220 nm and a GM-tube (Alnor mini-monitor, series 900).

^{1}H- and ^{13}C-NMR (90 MHz) were recorded using a JEOL FX90Q spectrometer and chemical shifts are reported in ppm downfield from internal tetramethylsilane (0.00 ppm) or from the known shift of the solvent used.

4-Trimethylammnoium-benzonitrile trifluoromethanesulfonate was synthesized from 4-(dimethylamino)benzonitrile using literature methods[12,13]; as intra m.p. 157–159°C (lit. 156–158°C[12]). ^{1}H-NMR (DMSO-d6) δ 3.65 (s, 9 H), 8.2 (s, 4 H).

4-Fluorobenzoic acid was produced by alkaline hydrolysis of the corresponding nitrile.[14] 4-Fluorobenzonitrile (6.06 g, 50.0 mmol) was heated to 110°C with NaOH (2.5 M, 90 ml) and refluxed for 1 h, until the liquid contained no oily drops and TLC (eluent $CHCl_3$; R_f acid $\approx$ 0.0–0.1, R_f nitrile $\approx$ 0.75) indicated that essentially all nitrile had been consumed. Trace amounts of starting material were removed by extraction with $CHCl_3$. HCl (12 M) was added to the aqueous phase until acidic pH. The white precipitate was filtered and dissolved in ethanol prior to azeotropic evaporation of the remaining water to give the product (6.5 g, 46.4 mmol, 93% yield); m.p. 181–183°C (182.6°C, according to the Merck Index). ^{1}H-NMR (MeOH-d4) δ 7.05–7.35 (m, 2 H), 7.95–8.2 (m, 2 H). ^{13}C-NMR (MeOH-d4) δ 115, 116, 133, 134, 162, 169, 173.

N-Succinimidyl 4-fluorobenzoate (SFB) was prepared from 4-fluorobenzoic acid, *N*-hydroxysuccinimide and DCC according to literature procedures[8]; m.p. 104–106°C (lit. 111–113°C[8]). ^{1}H-NMR ($CDCl_3$) δ 2.90 (s, 4 H), 7.05–7.30 (m, 2 H), 8.05–8.30 (m, 2 H).

N-4-Fluorobenzoyl-C-peptide (FB-C-peptide) was synthesized by reaction of C-peptide (2.25 mg, 0.745 μmol) with a 250-fold excess of SFB (43.5 mg, 0.1835 mmol) in a mixture of $NaHCO_3$ buffer (100 mM, pH 8.3) and MeCN (ratio 1:1, 4.5 ml). After reaction for 10 min, a hydroxylamine solution (pH 8.3) was added to a final concentration of 100 mM and reaction was continued for 10 min. The reaction mixture was diluted with water to a final MeCN concentration of 20% and clarified by centrifugation of 20 000 g for 10 min before FB-C-peptide was isolated by repeated HPLC preparations, using a Kromasil C8 column (4.6 × 250 mm, 7 μm). Elution was performed at 1 ml/min with a gradient of MeCN (containing 0.1% TFA) in dH_2O with MeCN increasing linearly from 20 to 42.5% during 15 min. The absorbance was monitored at 220 nm. Fractions containing FB-C-peptide were pooled,

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freeze-dried and stored in a freezer until use. The conversion, based on C-peptide and determined using HPLC, was 35%. The identity of FB-C-peptide was confirmed by matrix-assisted laser desorption ionization MS (MALDI Q-TOF): 3143 Da.

Radionuclide production

No-carrier-added $[^{18}F]F^-$ was produced via the $^{18}O(p,n)^{18}F$ reaction by bombardment of [^{18}O]water (94% enrichment, Rotem, diluted to 25-80% enrichment with 18 MΩ water from a Milli-Q filtration system, Barnstead) with 17 MeV protons, using the Scanditronix MC 16 cyclotron at the Karolinska Hospital/Institute. The produced $[^{18}F]F^-$ was either directly combined with K_2CO_3 (3.7 mg, 26.8 μmol) and Kryptofix® 2.2.2 (20 mg, 53.1 μmol), or, to recover the ^{18}O-enriched water, was first trapped on an anion exchange resin (Dowex (Cl^-) 1X-8, 200–400 mesh converted to OH-form according to Schlyer *et al.*[15]) and then eluted with K_2CO_3 (1.0 ml 0.03 M, 30 μmol) and combined with Kryptofix® 2.2.2 (22.6 mg, 60 μmol). In both cases, the water was subsequently evaporated to dryness, by azeotroping with MeCN under N_2 (g) at 120°C.

Synthesis of [^{18}F]SFB[9] from 4-[^{18}F]fluorobenzoic acid via 4-[^{18}F]fluorobenzonitrile[12]

The dried Kryptofix® 2.2.2/K^+ complex of $[^{18}F]F^-$ was resolubilized in DMSO (0.5 ml), added to 4-trimethylammoniumbenzonitrile trifluoromethanesulfonate (3.2 mg, 10.3 μmol, in a 10 ml vial) and reacted with stirring at 120°C for 10 min to produce 4-[^{18}F]fluorobenzonitrile. Radio-TLC (eluent $CHCl_3$) of an aliquot showed only one radioactive peak eluting with same R_f as 4-fluorobenzonitrile (R_f the nitrile ≈ 0.75, R_f $[^{18}F]F^-$ ≈ 0.0–0.1). 4-[^{18}F]Fluorobenzoic acid was produced by hydrolysis using NaOH (5 M, 1.0 ml) at 120°C for 10 min, with stirring. The acidified (HCl 12 M, 1.0 ml) and diluted solution (dH_2O 2.5 ml) was loaded onto a C18 Sep-Pak® (Waters, Sep-Pak® Plus). The Sep-Pak® was washed (HCl 0.012 M, 2.0 ml; then dH_2O 3.0 ml) before the product was eluted (MeCN 2.5 ml). According to radioanalytical HPLC (t_{Ret} ≈ 8.8 min), the 4-[^{18}F]fluorobenzoic acid had a radiochemical purity > 99%. Before drying the [^{18}F]fluorobenzoic acid by azeotropic evaporation with dry MeCN (3 × 1 ml, 95°C, N_2(g)), tetrabutylammonium hydroxide (20 μl, 25% in MeOH) was added.

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Thereafter *N*, *N*′-disuccinimidyl carbonate (5.3 mg, 20.7 μmol) in MeCN (0.3 ml) was added for reaction at 95°C for 10 min. The solvent was evaporated at r.t. with N_2 (g), the residue was dissolved in CH_2Cl_2 (0.25 ml) and purified by semi-preparative HPLC ($t_{Ret} \approx 14-15$ min). The [^{18}F]SFB was radiochemically pure (>99%) according to radio-analytical HPLC ($t_{Ret} \approx 13$ min).

Peptide labeling, [^{18}F]FB-C-peptide

After removing the HPLC eluent by rotary-evaporation, [^{18}F]SFB was dissolved in dry MeCN (200 μL) and transferred to a GC-vial (Scantec Lab, v-shaped vial, 1.5 ml). The solvent was evaporated at r.t. using N_2 (g) before dry MeCN (20 μl) was added. The vial was vortexed to facilitate the solubilisation before the peptide (0.8 mg (80 μl of a stock solution: pH 7.7–7.8; 10 mg peptide/ml 0.1 M borate buffer)) was added. After another vortexing, the sealed vial was placed in an ultrasonic bath at 55–60°C for 15 min, or until most of the [^{18}F]SFB had reacted, according to radio-HPLC. The [^{18}F]FB-C-peptide was isolated by semi-preparative HPLC ($t_{Ret} \approx 20-21$ min), rotary-evaporated to dryness, dissolved in physiologically-buffered saline, sterile-filtered (Millipore, Millex®-GV, 0.22 μm) and analyzed by coelution on HPLC with reference FB-C-peptide ([^{18}F]FB-C-peptide $t_{Ret} \approx 15$ min). The radio-chemical purity was greater than 99%.

Results and discussion

[^{18}F]SFB was synthesized by slight modifications of literature procedures.[8–11] Briefly, 4-trimethylammnoiumbenzonitrile trifluoro-methansulfonate was reacted with no-carrier-added [^{18}F]F^- to yield 4-[^{18}F]fluorobenzonitrile which was subsequently hydrolyzed to 4-[^{18}F]fluorobenzoic acid before reaction with *N*, *N*′-disuccinimidyl carbonate. [^{18}F]SFB was obtained in yields and reaction times that compare reasonably well with previous reports (for example, Reference 11). In four preparative runs, the decay-corrected yields of [^{18}F]SFB, were 24.4–29.2%, based on starting [^{18}F]F^- (up to 58%, based on resolubilized [^{18}F]F^-). The mean synthesis time, including HPLC isolation, was 155 min from resolubilized [^{18}F]F^-.

A typical chromatogram from the HPLC purification of [^{18}F]SFB is shown in Figure 2(a). Separation of [^{18}F]SFB from one other radiolabeled component and from a number of UV-absorbing unlabeled

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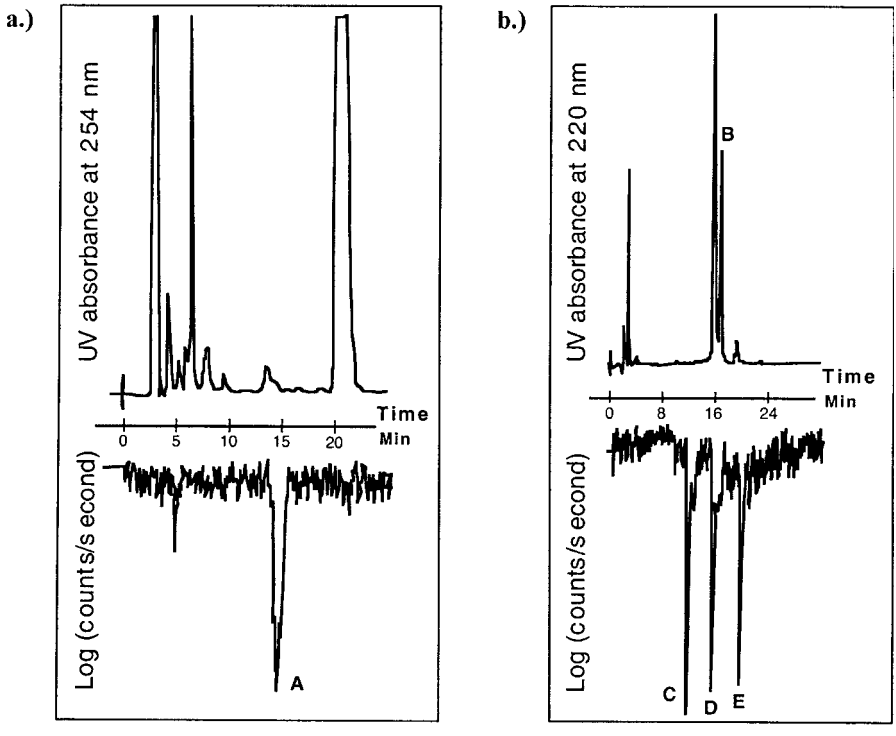

Figure 2. (a) HPLC chromatogram from isolation of [^{18}F]SFB, (peak A, $t_{Ret} \approx 14-15$ min. (b) HPLC chromatogram from isolation of [^{18}F]FB-C-peptide (peak E, $t_{Ret} \approx 20-21$ min). The UV peak B has the same retention time as C-peptide. Peak D eluted with the same retention time as 4-[^{18}F]fluorobenzoic acid, which may be formed by competing hydrolysis. One other labeled side-product (peak C) with a retention time less than that of the acid was also formed

components was clearly achieve. According to radioanalytical-HPLC, the radiochemical purity of the isolated [^{18}F]SFB was better than 99%. The recovery of [^{18}F]SFB in the four preparations was $80 \pm 6\%$ (mean $\pm$ std). Although the HPLC is time-consuming, the improvements in labeling yields obtained when [^{18}F]SFB is purified before the peptide-coupling reaction have been convincingly motivated elsewhere.[16]

Coupling optimization

In the conjugation labeling of C-peptide, the conversions of [^{18}F]SFB into the *N*-labeled peptide were initially low and varying. The results of the optimization experiments performed using aliquots of isolated [^{18}F]SFB are shown in Table 1.

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Table 1. Influence of pH and solvent on peptide labeling conjugation. Conversions are based on analysis by radioanalytical HPLC

pH	*n*	MeCN (%)	DMF (%)
7.5	1	18.2	20.8
7.7	3	21.0–25.4	14.9–19.4
8.4	2	6.9–10.1	7.5–10.9
8.7	1	5.2	4.6

The conjugation of [^{18}F]SFB with peptides suffers from competing side reactions. For example, [^{18}F]SFB is susceptible to hydrolysis yielding the labeled benzoic acid, which was also observed here (Figure 2(b)). In efforts to reduce the hydrolysis and to augment the solubility of [^{18}F]SFB and its contact with the peptide, we dissolved [^{18}F]SFB in either MeCN or DMF before addition of peptide solution (ratio 1:5, respectively) in the optimization experiments (Table 1). Both solvents were tolerated well and appreciable differences in conversions were only observed at pH 7.7.

The pH of the buffered peptide solution was found to be an important parameter for successful incorporation of the labeling agent. Over the range from physiological pH to pH 8.7, the yields were highest at pH 7.5–7.7, depending on the solvent used, and decreased rapidly as the pH was increased. At pH lower than 7.5, much smaller yields were obtained, which can be due to the increasing tendency for C-peptide to precipitate or gelate (p$I \approx 3.5$). In fact, when the TFA from the mobile phase in the HPLC isolation of [^{18}F]SFB was not completely removed, a cloudy suspension formed on adding the peptide solution and the coupling was unsuccessful. Best conversions of [^{18}F]SFB to [^{18}F]FB-C-peptide were obtained with MeCN at pH 7.7 and these conditions were used in the preparative runs.

The conjugation yields obtained here are somewhat lower than other labelings previously reported with [^{18}F]SFB (see Vaidyanathan *et al.*[11] and references therein). The yields were also quite low when the non-radioactive reference of FB-C-peptide was synthesized, despite attempts to drive the reaction by using large excesses of SFB and longer reaction times. Variations in labeling yields have previously been attributed to the homogeneity or heterogeneity of the mixtures.[11] The relatively small size and hydrophilic character of C-peptide may, therefore, make good contact with the lipophilic [^{18}F]SFB more difficult.

In four preparative runs, starting with 1150–1530 MBq of [^{18}F]SFB, 91–122 MBq of [^{18}F]FB-C-peptide was isolated. The decay-corrected

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yield of isolated [^{18}F]FB-C-peptide, counted from [^{18}F]SFB, ranged from 17.2 to 21.1%. After purification, the mobile phase was immediately evaporated and [^{18}F]FB-C-peptide was subsequently formulated in physiologically-buffered saline and sterile-filtered. The radiochemical purity of the labeled peptide was higher than 99%, according to HPLC analysis.

The specific radioactivity of the isolated labeled peptide should primarily be determined by that of the labeling agent, as long as the unlabeled peptide is efficiently separated from the product. In two of the production runs, the specific radioactivity of [^{18}F]SFB was estimated by radio-HPLC to be 175 and 260 GBq/μmol at end of synthesis (EOS) (or 680 and 880 GBq/μmol at end of bombardment (EOB)), based on 17.8 and 16.7 GBq starting [^{18}F]F$^-$, respectively. For the former of these two runs, the concentration of C-peptide in the final preparation was also estimated using ELISA (commercially available kits, Mercodia, Uppsala, Sweden). The specific radioactivity of [^{18}F]FB-C-peptide was calculated to be 70 at EOS or 590 GBq/μmol at EOB, which is in reasonably good agreement with the estimated specific radioactivity of [^{18}F]SFB at EOB (680 GBq/μmol). The specific radioactivity of the [^{18}F]FB-C-peptide, recalculated to EOB is consistent with those obtained in other radiolabelings performed with [^{18}F]F$^-$ produced with this target system.[17] For *in vivo* studies, an injected dose of 100 MBq would contain approximately 1.5 nmol peptide. Ambient physiological concentrations of C-peptide are 0.5–1.5 nM in healthy individuals and essentially zero in diabetic patients.[2] *In vivo* evaluation of the distribution of [^{18}F]FB-C-peptide in diabetic compared to healthy individuals, might reveal differences in the peptide's uptake, retention and elimination which can help explain the therapeutic effects observed in IDDM patients.

Conclusion

The conjugation labeling of C-peptide in the N-terminal with [^{18}F]SFB has been optimized. Using this method, [^{18}F]FB-C-peptide has been produced in decay-corrected yields of 4.2–6.1% ($n = 4$) starting from 14.1 to 17.8 GBq [^{18}F]F$^-$ and a total preparation time of around 5.5 h (or in 8–12% in less than 5 h counted from resolubilized [^{18}F]F$^-$). The times for individual reaction steps are comparable with those reported in the literature and the specific radioactivity of [^{18}F]FB-C-peptide was approximately 70 GBq/μmol at EOS. The amount of [^{18}F]FB-C-peptide

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obtained is sufficient for the PET studies, currently underway, of its distribution in humans.

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