Radiosynthesis of dimethyl-2-[18]F-(fluoromethyl)-6-methyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate for L-type calcium channel imaging

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Summary. Dimethyl 2-(fluoromethyl)-6-methyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate 4a, a fluorinated nifedipine analog, has been shown to elicit significant calcium channel blocker activity using a guinea pig ileal longitudinal smooth muscle model. In order to perform biological studies for detection of L-type calcium channel distribution, we decided to prepare the [18F]-labeled compound. The latter compound was prepared in no-carrier-added (n.c.a.) form from dimethyl 2-(bromomethyl)-6-methyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate 2 in one step at 80 °C in Kryptofix[222]/K[18F]F and acetonitrile as a solvent in 15 min. Column chromatography afforded the radiochemically pure compound in 20 min. Radiochemical purity of the [18F]-nifedipine was determined by RTLC and HPLC (> 98%) and specific activity of 21–48 GBq/μmol (EOB).

1. Introduction

1,4-dihydropyridines (DHPs), are primarily used for treatment of cardiovascular diseases such as hypertension, angina and some forms of cardiac arrhythmias. Recently, it has been suggested that these agents may be useful in other pathological states, including seizures and central ischemic disorders [1, 2]. Although these agents bind with high affinity to L-type Ca2+ channels, it has been known for many years that they also interact with non-L-type Ca2+ channel structures, such as nucleoside transporters, ATPases, and various other enzymes [3]. In addition, L-type Ca2+ channel blockers have been shown to block cation-selective ligand-gated ion channels, including N-methyl-D-aspartate (NMDA) receptors [4], nicotinic acetylcholine receptors [5, 6], and 5-HT3A receptors [7]. Thus, these agents do not selectively block voltage-gated ion channels.

An efficient radiosynthesis method has already been introduced for the preparation of 13C-dihydropyridines including nifedipine, by alkylation of the appropriate DHP 3-monocarboxylic acid anion with [11C]iodomethane, but

the method remains useful for incorporation of 11C-CH3 as an ester group [8]. In another study a carbon-11 labeled DHP, [11C]S12968, was used to measure the myocardial dihydropyridine binding site density using positron emission tomography [9–11]. Although carbon-11 is the best candidate for radiolabeling of biologically active compounds due to many pharmacological reasons, fluorine-18 is the mostly used radionuclide in PET preclinical and clinical studies, due to its physical properties and world-wide availability. Thus the production and evaluation of 18F-L-type calcium channel blockers remains of great interest.

Based on our previous works on the production and biological evaluation of fluorine-18 labeled compounds such as cholesteryl esters [12], benzodiazepine ligands [13] and with respect to our other research project on the interesting biological actions of dihydropyridines [14], we decided to prepare a 18F-labeled nifedipine analogue 4b. The synthesis utilizes commercially available nifedipine 1, to obtain, [18F]-labeled nifedipine tracer, through a simple and fast method in order to demonstrate calcium channel distribution in animal models (Fig. 1).

2. Experimental

2.1 Materials

The chemicals were purchased from Aldrich Chemical Company, Milwaukee, WI. All samples for NMR spectra were dissolved in CDCl3. 1H NMR spectra were run at 500 MHz, 13C NMR at 125 MHz, chemical shifts (δ) are reported in ppm relative to tetramethylsilane (δ 0.0, internal standard). For 13C NMR, chemical shifts are reported in ppm relative to CDCl3 (δ 77.0 for the central peak).

Mass spectra were recorded by a Finnigan Mat TSQ-70 Spectrometer. Thin-layer chromatography (TLC) of nonradioactive products was run on silica gel polymer-backed (F 1500/LS 254, 20 × 20 cm, TLC Ready Foils Schleicher & Schuell®) or glass plates (25 × 35 cm, E-Merck). Acetonitrile used for labeling experiments was of ‘Sure-Seal™’ grade (Aldrich). Analytical HPLC to determine the specific activity was performed by a Shimadzu™ LC-10AT, armed with two detector systems, flow scintillation ana-
of 1 mL: acetonitrile-water (60:40) was used as eluent at a flow rate of 1 mL/min (Rf = 5.48 min). The specific activity of 4b was calculated using a standard curve from 4a. Radiochromatography was performed by a AR-2000 Bioscan instrument, Paris, France, using polymer-backed silica gel papers.

Radiochemical purity and stability of [18F]-nifedipine was analyzed by TLC and reversed-phase HPLC. The identity of the product was confirmed by comparing radiochromatograms of [18F]-nifedipine with the UV-chromatograms of non-labelled reference material. Analytical HPLC was also used to determine the specific radioactivity of [18F]-nifedipine. A standard curve was generated to calculate the radioactivity of the product was confirmed by comparing radiochromatograms of [18F]-nifedipine with the UV-chromatograms of non-labelled reference material. Analytical HPLC was also used to determine the specific radioactivity of [18F]-nifedipine. A standard curve was generated to calculate the radioactivity of the final solution. The production of the labeled compound was performed in an automated fluorination module designed and manufactured locally. The purification of 4b was performed by C18 Sep-Pak™ short columns, which were purchased from Waters. Melting points were determined on a Reichert–Jung™ hot stage microscope and are uncorrected. All values were expressed as mean ± standard deviation (mean ± SD) and the data were compared using student T-test. Statistical significance was defined as P < 0.05.

2.2 Calcium channel blockade activity evaluation

The in vitro calcium channel antagonist activity (IC50) of [18F]-compound was determined as the molar concentration of the test compounds required to produce 50% inhibition of the high K+ concentration of guinea pig ileal longitudinal smooth muscle (GPILSM).

2.3 Preparation of [18F]-potassium fluoride

Fluorine-18 anion was prepared by 18 MeV proton bombardment of an enriched 18O-H2O sample (> 95%, Cortec™, France). The sample was held in a gold-coated target with a silver body in a 30 MeV cyclotron at our institution (AMIRS). After recovery of 18O-H2O over an anion cromafix PS HCO3 (Macherey-Nagel, Germany) Sep-Pak cartridge, fluorine-18 anion was eluted by a 1% potassium carbonate solution. The eluted solution was directly used in the labeling step.

2.4 Preparation of dimethyl 2-(bromomethyl)-6-methyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (2)

To the solution of 1 (0.33 g, 0.96 mmol) in dichloromethane (20.0 mL) at −10 °C was added pyridine (0.08 mL, 1 mmol) and pyridinium bromide perbromide (0.34 g, 1 mmol). The solution was stirred for 45 min (−20 °C) and after completion of the reaction, it was diluted with dichloromethane (20.0 mL) and washed with hydrochloric acid (2 × 20.0 mL of 2 M) and then with ice-water (2 × 20.0 mL). The solution was then dried over anhydrous sodium sulfate and the solvent was removed in vacuo without heating. The monobromide product (65%, m.p. > 135 °C decomposed) was obtained by column chromatography over silica gel (EtOAc/petroleum ether). 1H NMR (500 MHz, CDCl3): δ: 1.28 (3H, s, allylic CH3), 3.60 (3H, s, OCH3), 3.64 (3H, s, OCH3), 4.69 (1H, d, J = 11.5 Hz, CHBr), 4.78 (1H, d, J = 11.5 Hz, CH2Br), 5.76 (1H, s, CH), 6.18 (1H, broad, NH), 7.26–7.71 (4H, m, ArH); 13CN M R δ: 19.49, 27.28, 35.56, 51.17, 51.61, 103.48, 105.51, 124.04, 127.47, 130.91, 132.97, 141.03, 142.52, 144.89, 147.80, 166.60, 167.10.

2.5 Preparation of dimethyl 2-(fluoromethyl)-6-methyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (4a)

To the solution of 2 (0.850 g, 2 mmol) in dried tetrahydrofuran (10.0 mL) was added tetra-n-butyl ammonium fluoride (1.0 mL, 1 M in THF). The solution was stirred for 30 min (50 °C) and after completion of the reaction, the mixture was diluted by chloroform it was diluted with dichloromethane (20.0 mL) and washed with water (2 × 20.0 mL). The solution was then dried over anhydrous sodium sulfate and the solvent was removed in vacuo without heating. The product (10%, m.p. 129–131 °C) was obtained by column chromatography over silica gel (EtOAc/petroleum ether). 1H NMR (500 MHz, CDCl3): δ: 2.45 (3H, s, allylic CH3), 3.63 (3H, s, OCH3), 3.65 (3H, s, OCH3), 5.56–5.77 (2H, m, JHH = 15 Hz, JHF = 42.5 Hz, CH2F). 5.81 (1H, s, CH),
6.65 (1H, broad, NH), 7.31–7.75 (4H, m, ArH); 13C NMR δ: 20.00, 34.59, 51.59, 51.73, 83.04 (d, J = 169 Hz), 101.88, 104.91, 124.47, 127.77, 131.50, 133.32, 141.87, 144.38, 144.57 (d, J = 20 Hz), 148.28, 167.30, 167.67. Mass (m/z): 344 (M⁺, 5%), 347.2 (80%), 344 (100%), 300 (40%), 282 (35%), 59 (25%).

2.6 Preparation of dimethyl 2-methyl-4-(2-nitrophenyl)-6-((tosyloxy)methyl)-1,4-dihydropyridine-3,5-dicarboxylate (3)

To the solution of 2 (0.850 g, 2.0 mmol) in methanol (40.0 mL) at 0 °C was added sodium tosylate (5 g). The solution was stirred for 24 (0 °C) and after completion of the reaction (detected by TLC), the solvent was removed in vacuo. The product (45%, m.p. 121–125 °C) was obtained by column chromatography over silica gel (EtOAc/petroleum ether). 1H NMR (500 MHz, CDCl₃): δ: 2.42 (3H, s, allylic CH₃), 2.43 (3H, s, benzyllic CH₃), 3.39 (3H, s, OCH₃), 3.59 (3H, s, OCH₃), 4.91 (1H, d, CH₂OTs), 4.93 (1H, d, CH₂O T₄s), 5.63 (1H, s, CH), 6.88 (1H, broad, NH) 7.23–7.68 (8H, m, ArH). 13C NMR δ: 19.44, 21.68, 34.34, 51.19, 51.33, 55.25, 103.18, 107.86, 124.01, 127.45, 128.42, 129.68, 131.06, 132.91, 134.25, 136.24, 140.73, 145.31, 145.46, 147.73, 166.62, 167.07. Mass (m/z): 483 (45%), 357 (20%), 265 (20%), 92 (100%), 63 (50%).

2.7 Fluorination of dimethyl 2-(bromomethyl)-6-methyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (2)

with potassium fluoride and Kryptofix[222]

To a vial containing potassium fluoride (40 mg, 0.69 mmol) in water (0.5 mL) and Kryptofix[222] (200 mg, 0.54 mmol) in acetonitrile (1 mL), another acetonitrile aliquot was added (5 mL). The mixture was stirred and then dried by argon flow. Another portion of acetonitrile (5 mL) was added and dried again. The vial was cooled and a solution of 2 (76 mg, 0.18 µmol) in acetonitrile (5 mL) was added to the dried mixture. The vial was capped and heated to 80 °C in 15 min. After cooling, the mixture was mixed with water (25 mL) and passed through a C₁₈ column and washed with diethyl ether (10 mL). The organic layer was dried over anhydrous sodium sulfate and purified by preparative TLC silica gel on a polymer-backed silica gel layer eluted with a mixture of petroleum ether/ethyl acetate (50:40) as the mobile phase. The desired fluoride compound was separated (Rf = 0.7). The product was then confirmed to be 4a by comparing with the authentic sample previously prepared.

2.8 Preparation of [¹⁸F]-dimethyl 2-(fluoromethyl)-6-methyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (4b)

Compound 4b was prepared from 2 using the above procedure. A volume of target solution eluted with 100 µL of a 1% potassium carbonate (1 mg, 7 µmol) solution, containing 200 mCi of activity was transferred to a 2 mL vial containing Kryptofix[222] (10 mg, 0.027 mmol) and anhydrous acetonitrile (0.5 mL). The mixture was evaporated by slight heat and argon flow. Drying was repeated after addition of two more 0.5 mL portions of anhydrous acetonitrile. A mixture of 2 (25 mg, 59 µmol) in anhydrous acetonitrile (0.25 mL) was added to the dried residue and heated to 80 °C for 15 min. Diethyl ether (7 mL) was added to the residue and after mixing for 1 min, the solution mixture was passed through two Si Sep-Pak columns to remove possible amounts of fluoride anion and/or K₂₂₂. After cooling, the mixture was injected into the HPLC (Partisil-5 C₁₈, Whatman, JB271-1) instrument using a mixture of water/acetonitrile (60 : 40) as the mobile phase (flow rate: 1 mL/min). The fraction eluted at 5.48 min was recovered. The non radioactive precursor was eluted at 3.2 min and the presence of fluorinated compound was checked by co-injection of cold fluorinated precursor (4a, prepared in this study) resulting in a radiopeak with higher area under the curve. The remaining precursor in various runs ranged between 3%–7% (n = 7). The active solution fraction was checked for radiochemical purity by TLC on a polymer-backed silica gel layer eluted with a mixture of petroleum ether/ethyl acetate (60 : 40). The Rf values of ¹⁸F-fluoride and radiolabeled compound were 0.0 and 0.4 respectively. The TLC was repeated by co-spotting of ¹⁸F-compound as well as final active solution and it was shown that they migrate at the same Rf. Analytical reverse phase HPLC was performed using a mixture of water/acetonitrile (60 : 40) (flow rate: 1 mL/min, pressure: 130 KgF/cm²) showing one major single peak at Rf of 0.54 min. Both chromatography methods showed a high purity (greater than 98%) in the form of [¹⁸F]-nifedipine. The fraction eluted at 5.48 min was evaporated to dryness by the pressure of a nitrogen stream and slight heat and reconstituted in borate buffer (0.2 mL, pH = 8) followed by passing through a 0.22 mm filter for ultimate animal studies.

2.9 Stability of 4b in the final product

A sample of 4b (5 mCi) was kept at room temperature for 3 h while being checked by RTLC every half an hour. A sample (5 µL) was taken from the shaken mixture and the ratio of major radiopeak of the labeled compound to any other newly produced radiopeaks were checked by RTLC using a mixture of petroleum ether/ethyl acetate (60 : 40).

2.10 Serum stability studies

To 36.1 MBq (976 µCi) of 4b was added 500 µL of freshly prepared human serum and the resulting mixture was incubated at 37 °C for 3 h. Aliquots (5-µL) were analyzed by radio-TLC after 0, 0.25, 0.5, 1, 2 and 3 h of incubation to determine the stability of the complex in presence of human serum.

3. Results and discussion

3.1 Pharmacology

The in vitro calcium channel antagonist activities (IC₅₀) of compound 4a was determined as the molar concentration
of the test compounds required to produce 50% inhibition of the high K⁺ concentration of guinea pig ileal longitudinal smooth muscle (GPILSM), and are presented in Table 1. These results indicate that compound 4a exhibits less potent calcium channel antagonist activity than reference drug nifedipine (IC₅₀ = 0.5 ± 0.2 × 10⁻⁸ M), yet it is active at the nanomolar range. These data showed that production of radiofluorinated compound 4b can result in a suitable imaging tracer for L-type calcium channels.

3.2 Production of the precursors

With the generation of different ¹⁸F-radiopharmaceuticals, we were interested in labeling new series of dihydropyridine analogs due to fluorine-18 suitable half-life. According to our knowledge there is no ¹⁸F-labeled DHP compound reported in the literature.

We had already designed fluorination reactions in our lab with reduced total radiosynthesis time as well as automation studies for production of hundred millicuries amounts of research tracers [15]. Based on interesting biological data acquired in guinea pig studies for fluorinated nifedipine 4a, we tried to design appropriate radiolabeling precursors for the fluorination step. The methyl group on nifedipine molecule represents a suitable fluorination site in dihydropyridine series. The alkyl group in position 2 of the dihydropyridine ring is essential for the biological activity of DHPs. Due to Van Der Waals radius similarity of hydrogen and fluorine atoms the substitution of a fluorine atom instead of a hydrogen atom on one of the methyl groups of DHP ring seemed to be a suitable fluorination position.

In order to synthesize a fluorinated compound, production of a suitable active sulfonate ester on the CH₃ at the position 2 of the ring seemed possible.

The most studied radiofluorination precursors consist of tosylated and nosylated compounds and we have already produced some radiofluorinated compound starting from nosylated compounds in our labs [16]. In order to produce the nosylated precursor, i.e. dimethyl 2-methyl-4-(2-nitrophenyl)-6-((nosyloxy)methyl)-1,4-dihydropyridine-3,5-dicarboxylate, one approach was to prepare brominated compound. In the first step, the brominated compound was prepared in good yield at −40°C, the temperature was an essential factor in the yield of reaction.

Following the production of dimethyl 2-(bromomethyl)-6-methyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate 2, two possible reactions were performed. In the first reaction nosylated compound could be prepared using freshly produced silver nosylate salt and the bromo compound in anhydrous acetonitrile at room temperature and in the dark as previously reported in the literature [15].

However due to unknown reasons the nosylated compound was not separated from the reaction mixture, instead an unknown compound was separated with ¹H NMR pyridine ring pattern (data not shown). The oxidation of the ring can be a possible reason which could be explained by the presence of oxidizing silver cation in the reaction mixture.

Compound 3 was prepared using sodium salt of the nosyl anion and brominated compound and the structure was confirmed by ¹³C NMR, ¹H NMR and mass spectroscopy. Carrier compound 4a was prepared in two ways: a) Fluorination of the starting brominated compound using tetra-butyl ammonium fluoride, a well known fluorination agent, in order to obtain a standard authentic sample and b) the direct fluorination by addition of 2 (dissolved in CH₃CN) to an azeotropically dried mixture of Kryptofix[222] and potassium fluoride.

Separation of the fluorine-19 compound was performed by flash column chromatography on silica gel. ¹³C NMR, ¹H NMR spectroscopy and elemental analysis were used to characterize the fluorinated compound.

3.3 Radiofluorination of the precursors

The radiolabeled target molecule 4b was prepared according to Fig. 1. In a first try, the tosylated compound was used as the precursor in acetonitrile at 80–90°C. Further heating of the reaction mixture produced a more polar yellow compound which was not radiolabeled. Thus, the maximum temperature was assumed to be around 80°C. In none of our radiofluorination reactions starting tosylated compound any organic radiolabeled compound was detected using RTLC and HPLC.

The fact that the tosylate gave no reaction at all while the bromo derivative did, could be explained the formation of a hydrogen bridge between the hydrogen attached to the nitrogen and an oxygen of the tosylate group, increasing the energy difference with the transition state of the substitution reaction. This could perhaps be forwarded as a hypothesis, since the NMR samples were prepared almost at the same concentration (21 and 24 µmol in 1 mL of solvent). Note in this respect that the chemical shift of the N–H proton of the tosylate is distinctly different from those of the fluoro- and bromo-compound.

The other possible precursor was the starting brominated compound, this compound was unstable in light and solutions even in few hours and its solutions must be prepared shortly prior to the reaction. The brominated compound was radiofluorinated using above conditions in acetonitrile and 80°C since the degradation of the precursor was shown to give a yellow-colored unlabeled impurity as observed in tosylated precursor case.

The reaction mixture was then passed through Si SepPak columns in order to remove fluoride anion as well as other impurities.

The production of the labeled compound was confirmed by radioTLC and radioHPLC methods by comparison to cold fluorinated compound as reference peak in higher than 98% radiochemical purity. The fast eluting component on reverse phase was fluoride anion column (Rf = 0.8 min), while the late eluting compound at 5.48 min, was shown to be the radiolabeled compound. The final solution was evaporated.

Table 1. IC₅₀ values for various DHPs synthesized in this study.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MW</th>
<th>IC₅₀ ± SEM</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a</td>
<td>364</td>
<td>5.24 ± 4.5 × 10⁻⁸</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>425</td>
<td>2.05 ± 1.1 × 10⁻⁷</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>534</td>
<td>1.72 ± 1.1 × 10⁻⁸</td>
<td>4</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>346</td>
<td>0.5 ± 0.2 × 10⁻⁸</td>
<td>5</td>
</tr>
</tbody>
</table>
under a flow of nitrogen gas and warming at 50 °C and the residue was reconstituted in borate buffer solution (pH 8) for further biological studies (Fig. 2).

3.4 Serum stability of the radiolabeled compound

Incubation of compound 4b in freshly prepared human serum for 3 h at 37 °C showed the formation of radioactive species appearing 30–60 min after incubation. Further studies are needed to evaluate the nature of these species.

4. Conclusion

In this report, we used the substitution nucleophilic reaction conditions similar to those reported previously. However purification of labeled compound 4b from the starting brominated compound was performed by C18 preparative HPLC. In this study, 3%–7% of unlabeled compounds including precursor were observed upon HPLC analysis of the final purified product using UV detector. Fluorine-18 compound prepared from 2 was examined repeatedly by different chromatographic methods and showed a consistent final specific activity of 21–48 GBq/µmol (EOB). The reaction conditions (temperature, time, solvent and Kryptofix/base ratio) were optimized to get the best results. This study offers an efficient and rapid synthetic route to prepare fluorine-18-labeled dihydropyridines can be easily automated in order to synthesize large scales of [18F]-dihydropyridines. The latter compound was obtained in higher than 98% of radiocative and 93%–97% chemical purity. This is a general route to the production of all DHP compounds having 1 or 2 methyl groups on position 2 and/or 6 of the DHP rings.

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References

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Fig. 2. HPLC chromatogram of final radiolabeled solution on a reversed phase column using a water : CH3CN mixture (60 : 40) as eluent.