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The syntheses and *in vitro* biotransformation studies of [^{14}C]apixaban, a highly potent, selective, efficacious and orally bioavailable inhibitor of blood coagulation Factor Xa

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Apixaban is a potent inhibitor of blood coagulation Factor Xa in the late stages of development. [^{14}C]apixaban was synthesized with the 14C label in the two different lactam ring systems within the molecule for various *in vitro* and *in vivo* metabolism studies. A nine-step synthesis of [^{14}C]apixaban, 10**, with the label in the central lactam ring was completed in 14% overall yield. A second synthesis of [^{14}C]apixaban,** 14**, with the 14C label in the outer lactam ring was completed in three steps in a 14% overall yield. No significant differences were observed between the metabolite profiles of** 10 **and** 14**, [^{14}C]apixaban, from both rat and human hepatocyte or microsomal incubations.**

Keywords: apixaban; Factor Xa; microsomes; hepatocytes; ADME

Introduction

Thrombotic diseases are the leading cause of death among people living in developed countries. Although the oral anticoagulant warfarin is effective in both venous and arterial thrombosis, it suffers from a narrow therapeutic index, slow onset of therapeutic effects, several drug and dietary interactions, the need for monitoring and dose adjustments. Thus, the discovery and development of a highly potent, selective, efficacious and orally bioavailable inhibitor of blood coagulation Factor Xa as a replacement for warfarin would be extremely valuable.[1] Apixaban represents such an agent and the preparation of [^{14}C] apixaban was required for various preclinical and clinical studies. This paper describes the syntheses, purifications, analyses and characterizations of [^{14}C]apixaban and its use in microsomal and hepatocyte *in vitro* and *in vivo* metabolism studies.[2]

Experimental

General Experimental: All reagents used were of ACS grade or higher. Flash chromatography was performed using either a Biotage Flash Chromatography System or an AnaLogix BSR Solvent Pump System with silica gel cartridges. HPLC analyses for the syntheses were performed on an Agilent 1100 HPLC system including solvent degasser, pump, automated injector and variable UV detector connected to either an IN/US BetaRam Flow Detector with a 0.25 ml detector cell or a Berthold Scintillation Detector with a 0.5 ml detector cell. HPLC gradient 1 = Phenomenex Luna C18, 5 μm, 4.6 × 150 mm column. Mobile phase A = 0.05% TFA in water, mobile phase B = 0.05% TFA in acetonitrile, 0 min 95% A, 6 min 50% A, 11 min 95% B, 13 min 95% B, 15 min 95% A, UV detection at 254 nm. HPLC gradient 2 = Vydac protein and peptide C18, 4.6 × 250 mm column. Mobile phase A = 0.05% TFA in water, mobile phase B = 0.05% TFA in acetonitrile, 0 min 90% A, 16 min 90% B, 23 min 95% B, 25 min 90% A, UV detection at 254 nm. HPLC Gradient 3 = Phenomenex, Luna, C18 (2), 3 μm, 150 × 4.6 mm column with Brownlee, RP-18 C18, 3 μm, 15 × 3.2 mm guard column, column temperature = 30°C, autosampler temperature = 4°C, mobile phase A = 0.4% formic acid in water adjusted to pH 3.2 with NH_4OH, mobile phase B = acetonitrile, mobile phase C = MeOH, 0 min 100% A, 25 min 20% A and 80% B, 27 min 50% B and 50% C, 32 min 50% B and 50% C, 34 min 100% A, flowrate = 1.0 ml/min at 323 nm. HPLC Gradient 4 = Ace 3, C18, 3 μm, 150 × 4.6 mm column with Ace 3, C18, 3 μm, 10 × 3.0 mm guard column, column temperature = 35°C, autosampler temperature = ambient, mobile phase A = 0.4% formic acid in water, adjusted to pH 3.2 with NH_4OH, mobile phase B = acetonitrile, 0 min 100% A, 3 min 100% A, 5 min 90% A, 20 min 75% A, 50 min 75% A, 60 min 50% A, 65 min 100% B, 70 min 100% B, 72 min 100% A, flowrate = 0.7 ml/min, UV detection at 240 nm. Preparative HPLC condition 1: Phenomenex Luna 5 μm, 250 × 21.2 mm column.

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Mobile phase A = 0.1% TFA in water, mobile phase B = 0.1% TFA in acetonitrile, 0 min 70% A, 18 min 95% B, 22 min 95% B, 25 min 70% A, flowrate = 15 ml/min. UV detection at 254 nm. Preparative HPLC condition 2: Zorbax SB-C18 5 μm, 250 × 21.2 mm column. Mobile phase A = 0.05% TFA in water, mobile phase B = 0.05% TFA in acetonitrile, 0 min 90% A, 16 min 90% B, 23 min 95% B, 25 min 90% A, Flowrate = 9.9 ml/min, UV detection at 254 nm. LC/MS analysis was performed on a Finnigan LXQ Mass Spectrometer System. NMR spectra were recorded on a Bruker AVANCE II 300 MHz Spectrometer with an Ultrashield™ Magnet or a Varian Unity/Inova 300 MHz Spectrometer. Specific activities were performed gravimetrically. Liquid scintillation counting was performed on either a PerkinElmer Tri-Carb Model 2900Tr or a Wallac Model 1409 Liquid Scintillation Counter.

Materials and equipment used in microsomal and hepatocyte studies

Cryopreserved human hepatocytes (mixed gender, pooled from $n \geqslant 3$) and rats (Sprague-Dawley, mixed gender) hepatocytes were obtained from Celsis In Vitro Technologies, Inc. (Baltimore, MD). Liver microsomes from humans (mixed gender, pool of 50) and rats (Sprague-Dawley; pooled from mixed gender) were obtained from XenoTech, LLC (Kansas City, KS). Hepatocyte incubation media (InVitroGRO HI media), obtained from Celsis was used for hepatocyte incubations, and 0.1 M potassium phosphate buffer, pH 7.4, was used for microsomal incubations. 7-ethoxycoumarin (7-EC) and 7-hydroxycoumarin (7-HC) were obtained from Sigma-Aldrich (St. Louis, MO). Incubations were analyzed by HPLC with a Waters 2695 Separations Module with Waters 486 UV Detector or 996 PDA Detector with Millennium v 4.0 Data System. Deepwell Luna Plate™-96 was from PerkinElmer Company. SpeedVac Concentrator AES2010 was from Savant Instrument Inc. Fraction Collector Model ISCO Foxy 200 was from ISCO, Inc. Packard Topcount NXT™ Microplate Scintillation & Luminescence Counter was from the Packard Instrument Company. Radioactivity was measured with a β-RAM radioactivity detector, Model 3 from IN/US Systems Inc. and Liquid Scintillation Counters; Models LS 6000LL, LS 6000TA and LS 6000IC from Beckman Instruments, Inc.

1-(4-Nitrophenyl)piperidin-2-[^{14}C]one, 2

To a small flask was charged K_2CO_3 (200 mg, 1.45 mmol) in 0.30 ml water. To this was added 4-nitroaniline (276.3 mg, 2.00 mmol), THF (0.68 ml) and chlorobenzene (0.66 ml) resulting in a biphasic solution. To this was added [1-^{14}C]5-bromovaleryl chloride (346.2 mg, 1.72 mmol, 100 mCi, specific activity = 58.0 mCi/mmol, source = GE Healthcare) dropwise and the reaction was stirred at room temperature for 15 min. To the heterogeneous solution was added tetrabutylammonium bromide (TBAB, 12.8 mg, 0.040 mmol) and KOH (132 mg, 4.0 mmol, 85%) dissolved in 0.28 ml H_2O. Progress of the reaction was monitored by HPLC using gradient 1. Product retention time = 8.25 min. After 3 h, the organic solvents were removed under reduced pressure leaving the aqueous solution. The aqueous layer was extracted with EtOAc (2 × 15 ml). The pooled EtOAc extracts were washed with water, brine, dried over Na_2SO_4, filtered and solvent removed under reduced pressure to give 780 mg of crude product. The reaction was repeated on the same scale and the combined crude products were purified by flash chromatography on silica gel eluting with 10% EtOAc : 90% hexane to 100% EtOAc. The product eluted at 60–80% EtOAc. The solvent from the pooled fractions was removed under reduced pressure to give 766 mg, 200 mCi, (100% yield) of a light yellow solid. HPLC analysis of the product using gradient 1 showed the product to be 100% radiochemically pure. Co-injection of the radiolabeled product with an unlabeled standard of product **2** produced a single peak. LC/MS showed a single peak with *m/z* ESI (+) = 221(4%), 223(100%), 224(9%) as expected. ^{1}H-NMR (300 MHz, DMSO-D_6) δ ppm 8.30(*d*, *J* = 9.06 Hz, 2H), 7.68(*d*, *J* = 9.06 Hz, 2H), 3.78(*t*, *J* = 5.57 Hz, 2H), 2.49–2.54(m, 2H, partially buried under DMSO peak), 1.86–2.01(m, 4H).

3,3-Dichloro-1-(4-nitrophenyl)piperidin-2-[^{14}C]one, 3

To a dry flask was charged **2** (192 mg, 0.872 mmol), chlorobenzene (2.0 ml) and PCl_5 (599 mg, 2.88 mmol). The reaction mixture was heated to 53°C for 1.5 h at which time all of the solid PCl_5 had dissolved and the reaction was complete by HPLC analysis using gradient 1. The retention time of the product was 10.25 min. The reaction mixture was cooled to room temperature and water (3.0 ml) was added slowly while cooling in an ice water bath. The layers were separated and the aqueous layer was extracted with CH_2Cl_2 (2 × 10 ml). The CH_2Cl_2 extracts from a second reaction on the same scale were combined with the first and were washed with brine, dried over $MgSO_4$ filtered and solvent was removed under reduced pressure. Further drying on a vacuum line produced 397 mg (79% yield) of a yellow solid, **3**. The product was 98.6% radiochemically pure by HPLC using gradient 1. ^{1}H-NMR (300 MHz, DMSO-D_6) δ ppm 8.37(*d*, *J* = 8.88 Hz, 2H), 7.69(*d*, *J* = 8.88 Hz, 2H,) 3.90(*t*, *J* = 5.95 Hz, 2H), 2.98–3.06(m, 2H) 2.21(quin, *J* = 5.76 Hz, 2H).

3-Chloro-1-(4-nitrophenyl)-5,6-dihydropyridin-2(1H)-[^{14}C]one, 4

To a dry flask was charged **3** (391 mg, 1.35 mmol), LiCl (29.4 mg, 0.692 mmol) and DMF (1.1 ml). The reaction was warmed to 105°C for 20 min and Li_2CO_3 (54 mg, 0.73 mmol) was added in two equal portions over 40 min. The mixture was heated to 105°C for 4 h at which time HPLC analysis using gradient 1 showed the reaction to be 97% complete The retention time of product was 9.55 min. As the reaction cooled, a precipitate formed. At room temperature, water (1.5 ml) was added and the large solid particles were broken up with a spatula. The suspension was centrifuged at 3000 rpm for 10 min, the supernatant was decanted and this was repeated with another 1.0 ml of water, followed by washing with isopropyl alcohol (1.0 ml) to give an off-white solid. The aqueous washings were extracted with CH_2Cl_2 (2 × 10 ml). The combined CH_2Cl_2 extracts were washed with brine, dried over $MgSO_4$ and solvent removed under reduced pressure to give a solid that was combined with the solid from the iPrOH washings after solvent removal to give 341 mg of an off white solid (100% crude yield). The product was 96% radiochemically pure by HPLC using gradient 1. The product was used in the next step of the synthesis without additional purification.

Ethyl 1-(4-methoxyphenyl)-6-(4-nitrophenyl)-7-[^{14}C]oxo-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxylate, 6

To a dry flask was added **4** (40.0 mg, 0.158 mmol), ethyl-2-chloro-2-(2-(4-methoxyphenyl)hydrazono)acetate **5** (42.3 mg, 0.165 mmol) and toluene (0.346 ml). The suspension was heated to 96°C. To this was added Et_3N (46 μl, 0.33 mmol) in two equal portions over 20 min. The reaction continued to be heated at 96°C for 2 h

and then cooled to room temperature. A second batch starting with 300 mg of **4** was also completed. Both batches were combined. Water (10 ml) was added and the aqueous layer was extracted with CH_2Cl_2 (3 × 15 ml). The CH_2Cl_2 layers were combined, washed with brine, dried over $MgSO_4$, filtered and solvent removed under reduced pressure to give 433 mg of a light brown solid (74% crude yield). HPLC analysis using gradient 1 showed the product was 90% pure with a retention time = 11.33 - min. The product was used in the next step of the synthesis without additional purification.

Ethyl 6-(4-aminophenyl)-1-(4-methoxyphenyl)-7-[^{14}C]oxo-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxylate, 7

To a 30 ml high pressure glass vessel with stirbar was charged **6** (433 mg, 0.992 mmol), NMP (2.25 ml) and 43 mg of 5% Pd on Al_2O_3. The black mixture was purged with nitrogen gas for 15 min, then pressurized with hydrogen gas to 15 psi, pressure released and repeated four additional times. Finally, it was pressurized with hydrogen gas to 30 psi and then heated to 52°C for 30 min. The pressure dropped to 20 psi and the reaction was repressurized with hydrogen gas to 30 psi. After 1 h, the reaction showed only 5% product by HPLC using gradient 1. The retention time of 7 was 7.52 min. The reaction was stopped, the Pd was filtered away and the reaction was restarted with 43 mg of fresh 5% Pd on Al_2O_3. The vessel was repressurized with hydrogen gas to 30 psi using the same procedure as described above and then heated to 52°C. After 2 h, the reaction mixture was 88% radiochemically pure product **7** by HPLC. The reaction mixture was cooled to room temperature and filtered to remove the Pd. The resulting NMP solution was dried over $MgSO_4$, filtered and the NMP removed under vacuum to give 400 mg (99% yield) of product that was used in the next step without additional purification.

Ethyl 6-(4-(5-chloropentanamido)phenyl)-1-(4-methoxyphenyl)-7-[^{14}C]oxo-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxylate, 8

To a dry flask with stirbar under nitrogen was charged **7** (330 mg, 0.812 mmol) in NMP (4.0 ml). 5-Chlorovaleryl chloride was added slowly and stirred at room temperature for 15 min. HPLC analysis showed the formation of 83% of the amide product. The retention time of product **8** using HPLC gradient 1 was 10.98 min. The reaction was stirred for 15 additional minutes and water (8.0 ml) was added slowly to form a cloudy solution. The aqueous solution was extracted with CH_2Cl_2 (3 × 10 ml). The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered and solvent removed under reduced pressure resulting in a brown paste. The crude product was purified by flash column chromatography on silica gel using a gradient starting from 10:90 EtOAc:hexane to 100% EtOAc. Fractions containing the desired product were pooled and the solvent removed under reduced pressure to produce 200 mg of an off white solid (47% yield). HPLC analysis using gradient 1 showed the product to be 90% radiochemically pure. The product was used in the next step without additional purification. LC/MS analysis showed ESI (+) m/z = 525(5%), 527(100%), 529(35%). ^{1}H-NMR (300 MHz, DMSO-D_6) δ ppm: 10.03(*s*, 1H), 7.65(*d*, J = 8.31 Hz, 2H), 7.54(*d*, J = 8.88 Hz, 2H), 7.33(*d*, J = 8.31 Hz, 2H), 7.06(*d*, J = 8.88 Hz, 2H), 4.40(q, J = 7.20 Hz, 2H), 4.10(*t*, J = 6.80 Hz, 2H) 3.87(s, 3H), 3.73(*t*, J = 6.04 Hz, 2H) 3.25(*t*, J = 6.80 Hz, 2H) 2.40(*t*, J = 6.89 Hz, 2H) 1.80(m, 4H), 1.39(*t*, J = 7.10 Hz, 3H).

Ethyl 1-(4-methoxyphenyl)-7-[^{14}C]oxo-6-(4-(2-oxopiperidin-1-yl)phenyl)-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxylate, 9

To a dry small flask with stirbar under nitrogen was charged **8** (197 mg, 0.372 mmol), NMP (2.0 ml), triethylorthoformate (0.156 ml, 0.938 mmol) and TFA (5.6 μl, 0.075 mmol). The solution was stirred at room temperature for 25 min and 21% NaOEt in EtOH (0.350 ml, 0.938 mmol) was added slowly over 5 min. After 30 min, HPLC analysis using gradient 1 showed the reaction was complete. Water (7.0 ml) was added along with 3.5 mg crude product from a previous small scale reaction. The pH was adjusted with saturated NH_4Cl solution to pH 7. The mixture was extracted with CH_2Cl_2 (3 × 10 ml). The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered and the solvent removed under reduced pressure to produce a red paste. The crude product was purified by silica gel flash column chromatography using a gradient starting with 30:70 EtOAc: hexane to 100% EtOAc to 5:95 MeOH:EtOAc. Pure fractions were pooled and the solvent was removed under reduced pressure. The sample was further dried on a vacuum line to produce an oil. The oil was dissolved in ether (2.5 ml) and the ether was removed under reduced pressure. The resulting solid was dried on a vacuum line to produce 142 mg of a white solid (78% yield). The product was 99.1% radiochemically pure by HPLC using gradient 1. LC/MS showed ESI (+) m/z = 489(5%), 491(100%), 492(27.5%), 493(5%). ^{1}H-NMR (300 MHz, DMSO-D_6) δ ppm: 7.56 (*d*, J = 9.06 Hz, 2H), 7.39–7.45 (m, 2H), 7.32–7.38 (m, 2H), 7.07(*d*, J = 9.06 Hz, 2H), 4.41(q, J = 7.05 Hz, 2H), 4.15(*t*, J = 6.52 Hz, 2H), 3.87(*s*, 3H), 3.66(*t*, J = 5.38 Hz, 2H), 3.27(*t*, J = 6.42 Hz, 2H), 2.45(*t*, J = 6.04 Hz, 2H), 1.87–2.01(m, 4H), 1.40(*t*, J = 7.08 Hz, 3H).

1-(4-Methoxyphenyl)-7-[^{14}C]oxo-6-(4-(2-oxopiperidin-1-yl)-phenyl)-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxamide, 10, [^{14}C]apixaban

To a dry flask was charged **9** (20.4 mg, 0.042 mmol), DMF (0.15 ml) and formamide (46 μl, 1.2 mmol). The solution was warmed to 50°C and trimethylorthoformate (3.2 μl, 29 μmol) and TFA in DMF (2.0 μl of solution containing 0.61 μl TFA, 8.2 μmol) were added. After stirring for 15 min, NaOMe in MeOH (25 wt%, 18 μl, 78 μmol) was added and the solution stirred for 30 min. To the reaction at 50°C was added water (0.5 ml). The reaction was cooled to room temperature overnight. The suspension was centrifuged at 3000 rpm for 15 min and the supernatant removed. Water (0.5 ml) was added and centrifugation was repeated. The centrifugation step was repeated twice more with isopropyl alcohol. The solid product was dried under high vacuum to produce 10.1 mg of white solid, **10**. The water and isopropyl alcohol washes were recovered and were purified by preparative HPLC using gradient 1 to produce an additional 5.8 mg of product (83% yield, 14% overall yield). HPLC analysis using gradient 1 showed the product to be 99.3% radiochemically pure and 99.9% chemically pure at 254 nm. The specific activity was measured gravimetrically at 56.4 mCi/mmol or 122 μCi/mg. LC/MS showed ESI (+) 460(5%), 462(100%), 463(28%) and 464(5%). ^{1}H-NMR (300 MHz, DMSO-D_6) δ ppm: 7.72(*s*, 1H), 7.50(*d*, J = 8.88 Hz, 2H), 7.44(*s*, 1H), 7.32–7.38(m, 2H), 7.25–7.32 (m, 2H), 7.00(*d*, J = 8.88 Hz, 2H), 4.05(*t*, J = 6.42 Hz, 2H), 3.80(s, 3H), 3.60(*t*, J = 5.57 Hz, 2H), 3.20(*t*, J = 6.61 Hz, 2H), 2.39(*t*, J = 6.33 Hz, 2H), 1.80–1.91(m, 4H).

Ethyl 6-(4-(5-bromopentan-[^{14}C]amido)phenyl)-1-(4-methoxyphenyl)-7-oxo-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxylate, 12

To a flask with stirbar was charged **11** (432.7 mg, 1.065 mmol) in THF (7.0 ml) and 25% K_2CO_3 (aq) (0.90 ml). This material was sonicated to completely dissolve all solids and form a pale brown solution. To this was added [1-^{14}C]5-bromovaleryl chloride (50 mCi, 51 mCi/mmol, in 2 ml THF, >98% radiochemically pure, source = ViTrax) dropwise and then it was stirred overnight at room temperature. The THF was removed under reduced pressure. Sterile water (20 ml) and CH_2Cl_2 (20 ml) were added. The two layers were separated. The water layer was extracted with CH_2Cl_2 (20 ml). The CH_2Cl_2 extracts were dried over Na_2SO_4, filtered and solvent removed under reduced pressure. To the residue was added EtOAc (20 ml) to form a solid. The solid was collected by filtration and dried under full vacuum overnight to give 330 mg of product (54% yield). HPLC analysis using gradient 2 showed the product to be >99% radiochemically pure. The product was used in next step without additional purification. A second reaction was performed on the same scale to give an additional 510 mg of product (84% yield) for an average yield of 69%.

Ethyl 1-(4-methoxyphenyl)-7-oxo-6-(4-(2-[^{14}C]oxopiperidin-1-yl)phenyl)-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxylate, 13

To a dry small flask with stirbar was charged **12** (330 mg, 0.581 mmol) and THF (3.0 ml). To this slurry was added KOEt (24% in EtOH, 0.30 ml) dropwise and then the reaction was stirred for 5 h. The reaction was acidified with glacial acetic acid (0.10 ml). The solvent was removed under reduced pressure and the residue was partitioned between water (10 ml) and CH_2Cl_2 (10 ml). The aqueous phase was extracted with CH_2Cl_2 (3 × 10 ml). The combined CH_2Cl_2 extracts were dried over Na_2SO_4, filtered and the solvent removed under reduced pressure to give an oil. The oil was taken up in EtOAc (1 ml) and left overnight to induce crystallization. The solid was collected by filtration to give 150 mg (53% crude yield) of solid product **13**. HPLC analysis using HPLC gradient 2 showed the product to be 88% radiochemically pure and 90% pure by UV area % at 254 nm. A second reaction starting with 510 mg of **12** was completed. The crude product was purified by silica gel flash chromatography eluting with 4% MeOH and 96% CH_2Cl_2 in place of the crystallization with EtOAc to produce 180 mg (41% crude yield) of product **13**. HPLC analysis of the second batch using gradient 2 showed the product to be 94% radiochemically pure with a UV area % purity of 91% at 254 nm. The crude products from both reactions were combined and subjected to preparative HPLC using gradient 2. Pure fractions were pooled and the acetonitrile was removed under reduced pressure. The remaining aqueous layer was extracted with CH_2Cl_2 (3 × 100 ml) and then with EtOAc (2 × 100 ml). The combined organic extracts were dried over Na_2SO_4, filtered and the solvent removed under reduced pressure to give 250 mg of a white solid, (35% yield). The product was >99.9% radiochemically pure and >99.9% pure by UV area % at 254 nm using gradient 2.

1-(4-Methoxyphenyl)-7-oxo-6-(4-(2-[^{14}C]oxopiperidin-1-yl)-phenyl)-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxamide, 14 or [^{14}C]apixaban

To a pressure tube was added product **13** (250 mg, 0.512 mmol) and a saturated solution of ammonia in 1,2-propanediol (4.5 ml) prepared by bubbling ammonia gas through 1,2-propanediol for 20 min. The reaction was heated to 120°C for 16 h, cooled to room temperature and diluted with water (20 ml). The aqueous solution was extracted with CH_2Cl_2 (3 × 20 ml) and then with EtOAc (3 × 20 ml). The combined organic extracts were dried over Na_2SO_4, filtered and the solvent removed under reduced pressure to give a solid. To the solid was added acetonitrile (5 ml) and the mixture warmed to 80°C to produce a clear solution. After cooling to room temperature over an hour, a solid formed. The sample was filtered and dried to give 134 mg of a white solid, (57% yield, 14% overall yield). HPLC analysis using gradient 3 showed the sample was 100% radiochemically pure. The specific activity of [^{14}C]apixaban, **14**, was measured gravimetrically at 49.5 mCi/mmol or 108 μCi/mg. The specific activity of the product was reduced to 2.49 mCi/mmol or 5.4 μCi/mg with unlabeled apixaban for use in the human ADME clinical study.[3] ^{1}H-NMR (300 MHz, $CDCl_3$) δ ppm: 7.49(*d*, *J* = 8.80 Hz, 2H), 7.37(*d*, *J* = 9.10 Hz, 2H), 7.26(*d*, *J* = 8.80 Hz, 2H), 6.98(*s*, 1H), 6.95(*d*, *J* = 9.20 Hz, 2H), 6.28(*s*, 1H), 4.14(*t*, *J* = 6.60 Hz, 2H), 3.81(*s*, 3H), 3.61(m, 2H), 3.39(*t*, *J* = 6.60 Hz, 2H), 2.63(*t*, *J* = 6.20 Hz, 2H), 1.96(m, 4H).

Incubations

As positive controls, 7-ethoxycoumarin (7-EC) and 7-hydroxycoumarin (7-HC) were dissolved in methanol. The solutions were separately spiked to cell suspension and liver microsomes with a final concentration of ca. 100 μM (containing 1% methanol). The mixtures were incubated for 1 and 4 h in a manner similar to apixaban incubations. At 0 and 4 hr, an aliquot of the incubation mixtures was extracted with two volumes of ice-cold methanol, followed by centrifugation. The resultant extracts were analyzed by HPLC using gradient 3. At the completion of a 4-hour incubation, 83 and 92% of cells remained viable for rat and human hepatocytes, respectively. After 4-hour incubation, 44.1 and 51.0% of 7-EC and 42.1 and 97.8% of 7-HC were metabolized by rat and human hepatocytes, respectively. In addition, significant amounts of 7-HC sulfate and 7-HC glucuronide were also observed. After 1-hour incubation, 71.2 and 90.2% of 7-EC was metabolized by rat and human liver microsomes, respectively. A significant amount of 7-HC, a metabolite of 7-EC, was also observed in 1-hour incubation samples.

As negative controls, [^{14}C]apixaban **10** at 122 μCi/mg and **14** at 108 μCi/mg, both 1 and 10 μM, were separately incubated with the incubation media for 1 and 4 h without enzyme systems. At the end of incubations, the incubations were extracted with two volumes of ice-cold acetonitrile, followed by centrifugation.

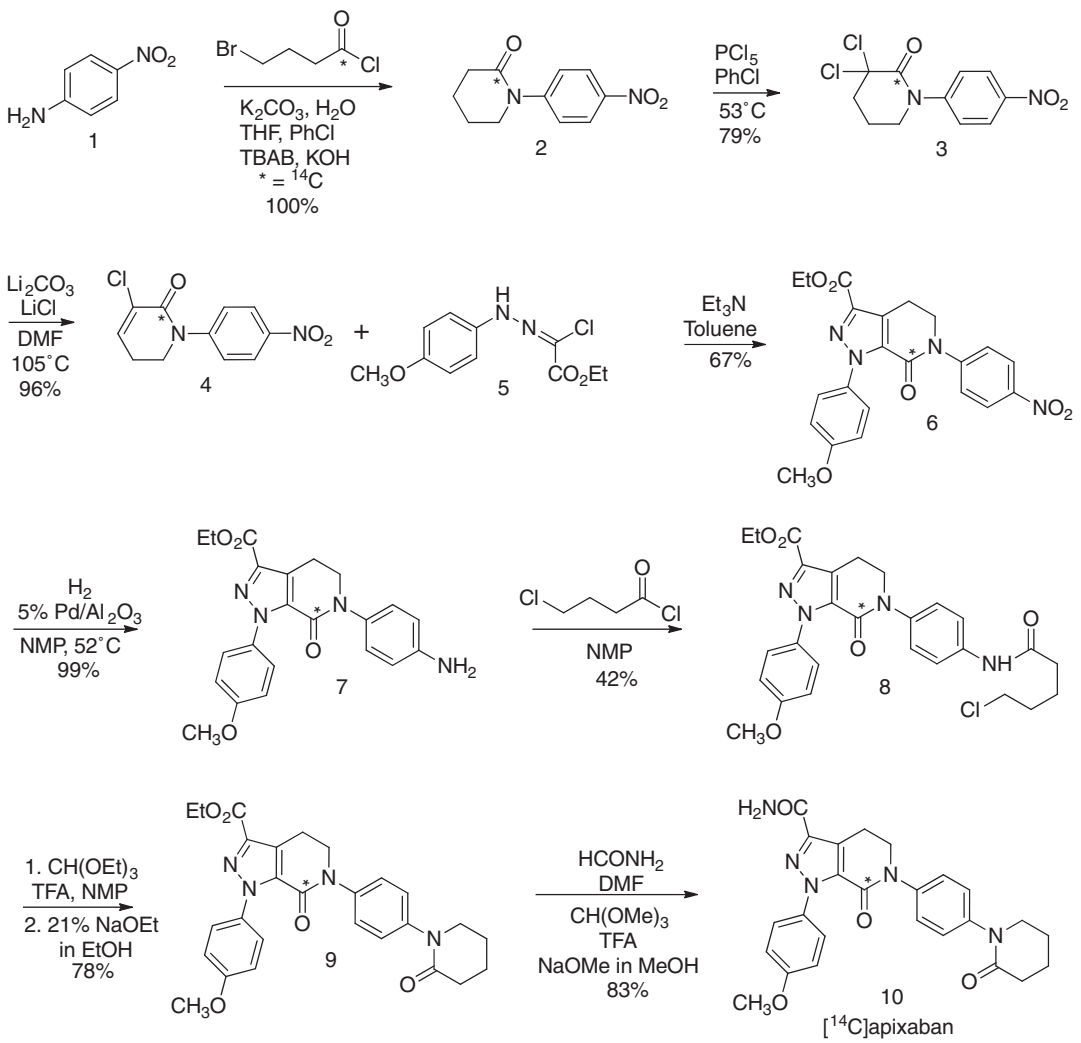
The cell viability was checked by the trypan blue exclusion method. [^{14}C]apixaban (both **10** and **14**), at 1 and 10 μM in incubation media, were separately incubated with cryopreserved rat and human hepatocyte suspensions (ca. 1.0×10^6 viable cells/ml) or 1 mg/ml liver microsomes in the presence of 5 mM $MgCl_2$ and 1 mM NADPH in 0.1 M potassium phosphate buffer (pH 7.4). Incubations were carried out in a 24-well cell culture plates with each well containing 0.5 ml of the incubation mixture. At 1, and 4 h, the entire incubation was quenched and extracted with 2 volumes of ice-cold acetonitrile. The supernatants from the incubation mixtures were concentrated using an N-Evap to dryness, and reconstituted with a mixture of acetonitrile/water (2:1) prior to analysis by HPLC. The 14C profiles in microsomal and hepatocyte incubation samples were

determined by HPLC radiochromatography using HPLC Gradient 4. Fractions of chromatography effluents were collected by time (15 s/fraction) to Deepwell LumaPlate™-96 plates. The plates were subsequently dried by a SpeedVac concentrator for up to 8 h. The radioactivity in each fraction was determined by Packard TopCount NXT™ Microplate Scintillation and Luminescence Counter technology. HPLC radiochromatograms were reconstructed using ARC Convert and Evaluation software. Radioactivity peaks were integrated to determine the percent distribution of individual radioactivity peaks or regions in each sample. Selected incubation sample extracts from microsomal and hepatocyte incubations were analyzed using HPLC gradient 4. The eluate was collected and the total volume was measured. Duplicate 2.0 ml aliquots of each HPLC eluate collection were taken for LSC. The data obtained with or without the use of an HPLC column were compared.

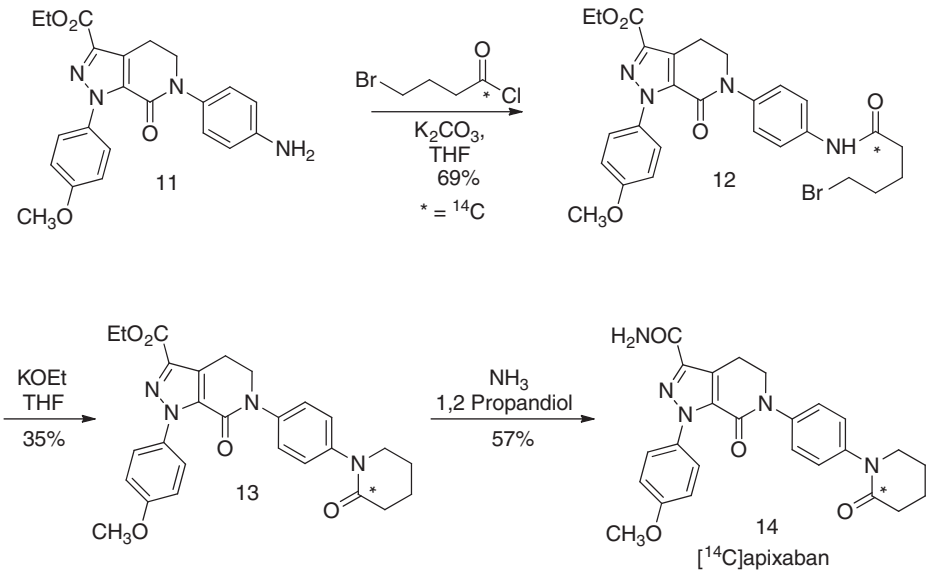
Results and discussion

[^{14}C]Apixaban was synthesized with the 14C label located in two different positions in the molecule. In the first synthesis shown in Scheme 1, 4-nitroaniline was reacted with [1-^{14}C]5-bromovaleryl chloride under basic conditions to generate nitro lactam **2** in quantitative yield. Product **2** was reacted with PCl_5 to produce α,α- dichlorinated lactam **3** in 79% yield. Product **3** was reacted with Li_2CO_3 at 105°C to produce α,β-unsaturated lactam **4** in 96% yield. Product **4** was reacted with ethyl 2-chloro-2-(2-(4-methoxyphenyl)hydrazono)acetate **5** synthesized by the Bristol-Myers Squibb Process Chemistry Group to generate the pyrazole ring system product **6**, in 67% yield. The aromatic nitro group was reduced to aniline **7** with hydrogen gas and 5% Pd on Al_2O_3 in 99% yield. The second lactam ring was prepared in four steps by reacting aniline product **7** with chlorovaleryl chloride to generate amide **8** in 42% yield followed by base promoted lactam ring formation in 78% yield. The final step involved the conversion of ethyl ester **9** to the primary amide with formamide to generate [^{14}C]apixaban, **10**, in 83% yield with the 14C label located in the central lactam ring system. The overall yield for the nine-step synthesis was 14%. The product was 99.3% radiochemically pure and 99.9% chemically pure by UV at 254 nm. MS and ^{1}H-NMR matched those expected for [^{14}C]apixaban. The specific activity was measured gravimetrically at 56.4 mCi/mmol or 122 μCi/mg.

A separate synthesis of [^{14}C]apixaban with the 14C label in the outer lactam ring system was completed and is shown in Scheme 2. In this synthesis, compound **11** synthesized by the



Scheme 1. Synthesis of **10**, [^{14}C]apixaban.



Scheme 2. Synthesis of **14**, [^{14}C]apixaban.

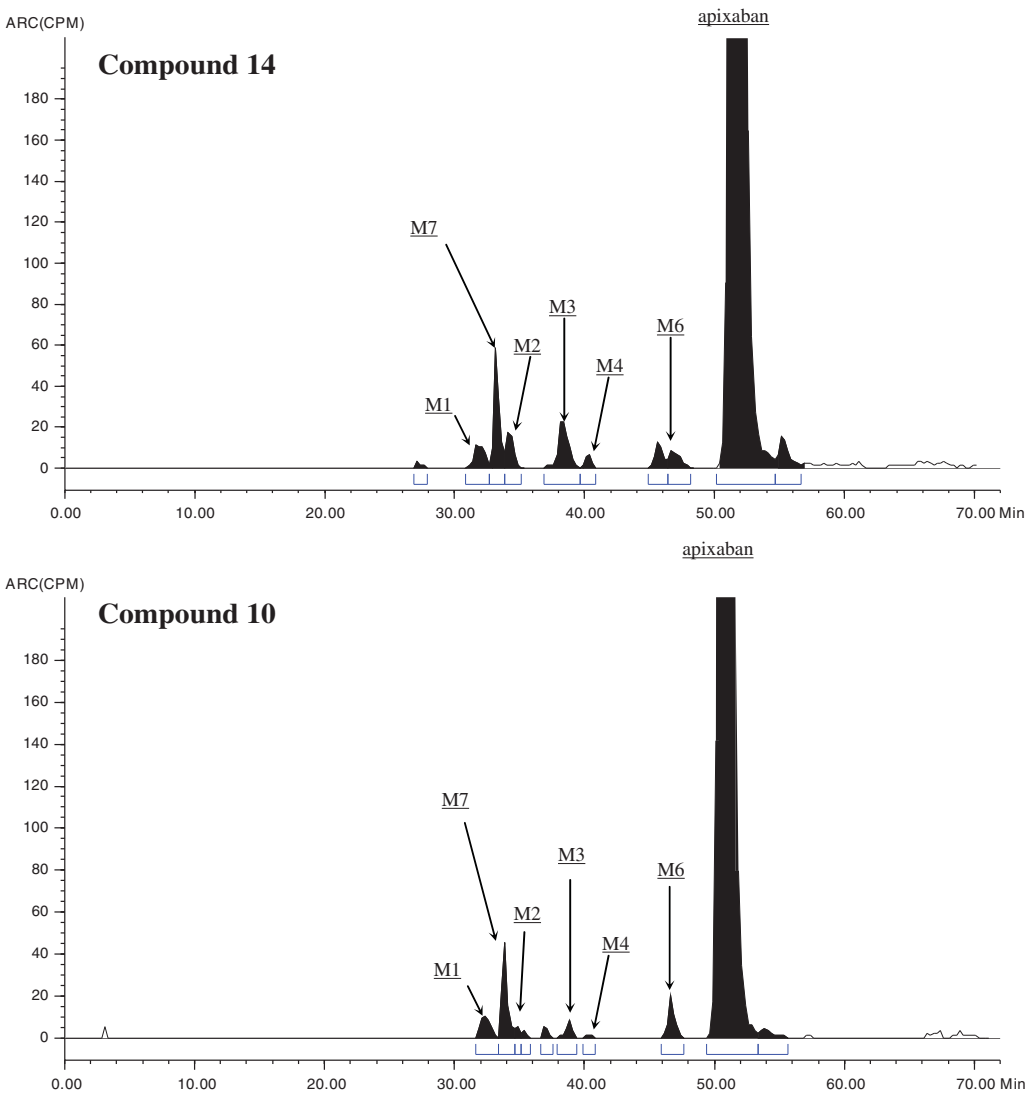


Figure 1. HPLC radiochromatograms of 4-hour human hepatocyte incubations with 10 μM [^{14}C]apixaban, compounds **10** and **14**.

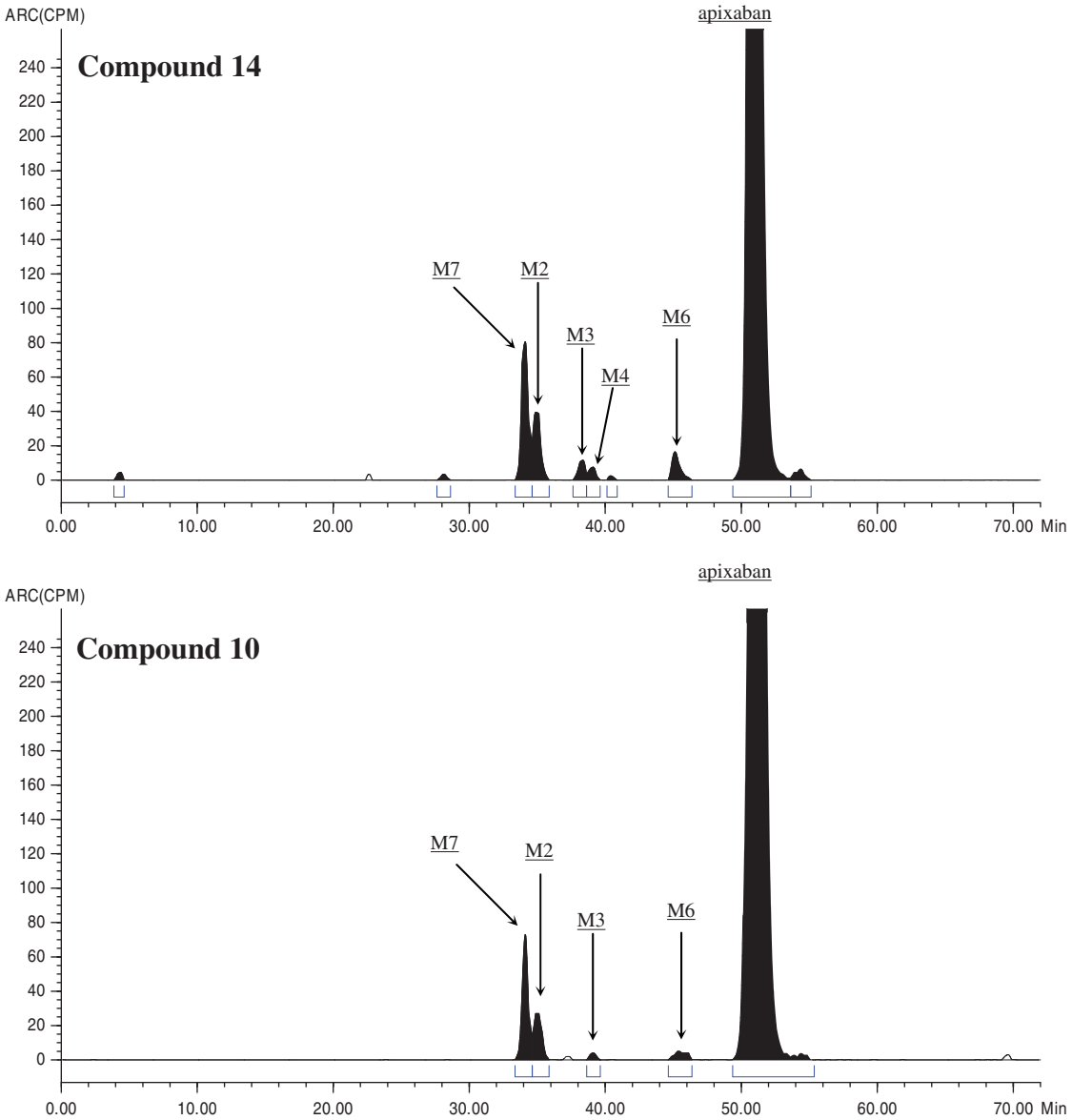


Figure 2. HPLC radiochromatograms of 1-hour human liver microsomal incubations with 10 μM [^{14}C]apixaban, compounds **10** and **14**.

Bristol-Myers Squibb Process Chemistry Group was reacted with [1-^{14}C]bromovaleryl chloride to generate amide **12** in 69% yield. Product **12** was converted with potassium ethoxide to the penultimate lactam **13** in 35% yield after purification by preparative HPLC. The penultimate ester was converted to [^{14}C]apixaban, **14**, with ammonia in 57% yield. The three-step sequence was completed in 14% overall yield. The final product was 100% radiochemically pure. MS and ^{1}H-NMR matched those expected for the labeled product. The specific activity of [^{14}C]apixaban, **14**, was measured at 49.5 mCi/mmol or 108 μCi/mg. The specific activity of a portion of the product was reduced to 2.49 mCi/mmol or 5.4 μCi/mg with unlabeled apixaban for use in the human ADME clinical study.[3]

The *in vitro* biotransformation profiles of both labels, **10** and **14**, in cryopreserved hepatocytes and liver microsomes from rats and humans were determined. Both hepatocyte preparations used in this study were enzymatically active for the 4-hour incubation. Also both microsomal preparations used in this study were enzymatically active for the 1-hour incubation.

The 1- or 4-hour microsomal or hepatocyte incubation mixtures of [^{14}C]apixaban (both **10** and **14**, at 1 and 10 μM), were extracted with ice-cold acetonitrile. The extraction recoveries of the total radioactivity from the incubation mixtures were quantitative for rat and human hepatocyte and microsomes for **14** and **10**. [^{14}C]apixaban **(**both **10** and **14**) appeared to be relatively stable in hepatocyte incubation media and phosphate buffer at 10 μM. Greater than 97% of [^{14}C]apixaban remained unchanged after a 1- or 4-hour incubation control. No detectable degradation products were observed in the 1 μM negative control incubations of both **14** and **10**.

The metabolism of apixaban in rat and human hepatocyte or microsomal incubations was very limited, with $>$92 and 95% of the total radioactivity being attributed to apixaban after a 1- or 4-hour incubation. Six metabolites, M1–M4, M6 and M7, were tentatively identified in hepatocytes and five metabolites, M2–M4, M6 and M7 were tentatively identified in microsomal incubations. These *in vitro* metabolites were similar to the *in vivo* metabolites.[3–5] Structures for all of the metabolites have been

reported previously in references 4 and 5. M2 was identified as the *O*-desmethyl metabolite.[4,5] M1 was a sulfate conjugate of M2.[4,5] M4 and M7 were hydroxylated metabolites.[4,5] M3 was the ring-opened metabolite[4] and M6 was the amide hydrolysis metabolite.[4] There were no significant differences observed between the metabolite profiles of both **10** and **14**, [^{14}C]apixaban, from both rat or human hepatocyte or microsomal incubations, see Figures 1 and 2. Metabolite profiles were similar for 1 and 10 μM incubations. Similar metabolic profiles of **10** and **14** supported the use of **14** for ADME studies and that experiments with compound **14** adequately assessed the metabolic profile of apixaban.

Conclusion

Two separate syntheses of [^{14}C]apixaban, **10** and **14**, labeled in two different lactam ring systems within the molecule were completed for various *in vitro* studies and *in vivo* studies. There were no significant differences observed between the metabolite profiles of **10** and **14** [^{14}C]apixaban from both rat or human hepatocyte or microsomal incubations.

Acknowledgements

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