

Supporting Information (SI)

Synthesis, Radiolabeling and Characterization of Plasma Protein-Binding Ligands: Potential Tools for Modulation of the Pharmacokinetic Properties of (Radio)Pharmaceuticals

Cristina Müller*^{1,2}, Renáta Farkas¹, Francesca Borgna³, Raffaella M. Schmid¹, Martina Benešová^{1,2},
Roger Schibli*^{1,2}

¹ Center for Radiopharmaceutical Sciences ETH-PSI-USZ, Paul Scherrer Institut, 5232 Villigen-PSI, Switzerland

² Department of Chemistry and Applied Biosciences, ETH Zurich, Vladimir-Prelog-Weg 1-5/10, 8093 Zurich, Switzerland

³ Department of Pharmaceutical and Pharmacological Sciences, University of Padua, Via Marzolo 5, 35131 Padova, Italy

E-Mail addresses:

cristina.mueller@psi.ch; renata.farkas@psi.ch; francesca.borgna@phd.unipd.it;
raffaella.schmid@psi.ch; martina.benesova@psi.ch; roger.schibli@psi.ch,

*** Correspondence to:**

PD Dr. Cristina Müller
Center for Radiopharmaceutical Sciences ETH-PSI-USZ
Paul Scherrer Institut
5232 Villigen-PSI
Switzerland
e-mail: cristina.mueller@psi.ch
phone: +41-56-310 44 54; fax: +41-56-310 28 49

Prof. Dr. Roger Schibli
Center for Radiopharmaceutical Sciences ETH-PSI-USZ
Paul Scherrer Institut
5232 Villigen-PSI
Switzerland
e-mail: roger.schibli@psi.ch
phone: +41-56-310 28 37; fax: +41-56-310 28 49

Table of Content

1. Synthesis of Plasma Protein Binding Entities
2. In Vitro Properties of the Radioligands
3. Stability of the Radioligands

1. Synthesis of Plasma Protein Binding Entities

4-(4-Iodophenyl)butyric acid (PPB-01). The compound (Figure S1) was purchased from Sigma-Aldrich, Germany.

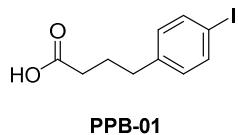
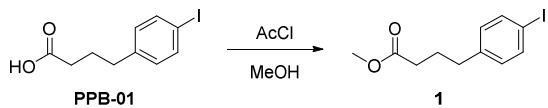


Figure S1: Chemical structure of 4-(4-iodophenyl)-butyric acid, herein referred to as PPB-01.

Organic Synthesis of 4-(4-Ethynylphenyl)butanoic acid (PPB-02). Synthesis of Methyl-4-(4-iodophenyl)butanoate (1) (Scheme S1). To a solution of 4-(4-iodophenyl)butyric acid (PPB-01) (205 mg, 0.71 mmol, 1 equiv) in MeOH (5 mL) acetyl chloride (0.5 mL, 6.31 mmol, 8.88 equiv) was added dropwise at 0 °C. The solution was allowed to reach room temperature and stirred for another 2 h. MeOH was removed under vacuum to give **1** in form of a yellow oil. Yield: 213 mg, 0.70 mmol, 99%. ¹H NMR (400 MHz, CDCl₃, δ, ppm): 7.60 (d, *J* = 8.4 Hz, 2H), 6.93 (d, *J* = 8.4 Hz, 2H), 3.66 (s, 3H), 2.59 (t, *J* = 7.6 Hz, 2H), 2.32 (t, *J* = 7.6 Hz, 2H), 1.93 (m, 2H).

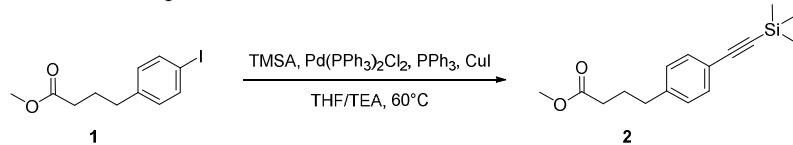
Scheme S1: Synthesis of compound **1**.



Synthesis of Methyl 4-((trimethylsilyl)ethynyl)phenylbutanoate (2) (Scheme S2). Under inert atmosphere compound **1** (213 mg, 0.70 mmol, 1 equiv) was dissolved in a mixture of tetrahydrofuran/triethylamine (1.5 mL, 2:1, v/v) and trimethylacetylene (TMSA, 120 μL, 0.85 mmol, 1.20 equiv), Pd(PPh₃)₄Cl₂ (9.8 mg, 14 μmol, 0.02 equiv), triphenylphosphine (PPh₃, 7.30 mg, 28 μmol, 0.04 equiv) and CuI (267 mg, 1.40 mmol, 2 equiv) were added. It was stirred at 60 °C for 16 h. The suspension was cooled to room temperature and filtered through celite. After the evaporation of the solvents the residue was purified by column chromatography using 5% ethyl acetate in hexane as

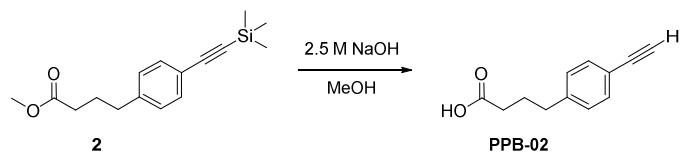
eluent to obtain compound **2** as amber oil. Yield: 176 mg, 0.64 mmol, 92%. ^1H NMR (400 MHz, CDCl_3 , δ , ppm): 7.38 (d, J = 8.4 Hz, 2H), 7.10 (d, J = 8.4 Hz, 2H), 3.65 (s, 3H), 2.64 (t, J = 7.6 Hz, 2H), 2.31 (t, J = 7.4 Hz, 2H), 1.97 – 1.90 (m, 2H), 0.24 (s, 9H). HRMS calculated for $\text{C}_{16}\text{H}_{23}\text{O}_2\text{Si}$ ($\text{M}+\text{H}$) $^+$ 275.1467, found 275.1461.

Scheme S2: Synthesis of compound **2**.



Synthesis of 4-(4-Ethynylphenyl)butanoic acid (PPB-02) (Scheme S3). Compound **2** (176 mg, 0.64 mmol) was dissolved in 1.5 mL MeOH and 1.5 mL 1 M NaOH was added and solution was stirred at 50°C for 2 h. The reaction mixture was acidified by addition of 1 M HCl and the product was extracted by DCM (3×5 mL) and dried over MgSO_4 . The solvent was removed under reduced pressure to give 4-(4-ethynylphenyl)butanoic acid (PPB-02) as a beige solid. Yield: 102 mg, 0.54 mmol, 85%. ^1H NMR (400 MHz, CDCl_3 , δ , ppm): 7.42 (d, J = 8.2 Hz, 2H), 7.14 (d, J = 8.2 Hz, 2H), 3.04 (s, 1H), 2.68 (t, J = 7.6 Hz, 2H), 2.37 (t, J = 7.4 Hz, 2H), 2.00 – 1.91 (m, 2H). HRMS calculated for $\text{C}_{12}\text{H}_{11}\text{O}_2$ ($\text{M}-\text{H}$) $^-$ 187.0765, found 187.0763.

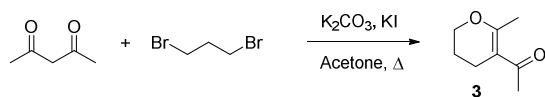
Scheme S3: Synthesis of 4-(4-ethynylphenyl)butanoic acid (PPB-02).



Organic Synthesis of 4-(3-(3,5-dimethyl-1H-pyrazol-4-yl)propoxy)-5-(methoxycarbonyl)phenoxybutanoic acid (MePPB-03). Synthesis of 3,4-dihydro-6-methyl-2H-pyran-5-yl methyl ketone (3) (Scheme S4). A mixture of pentane-2,4-dione (1.33 mL, 13 mmol, 1 equiv), K_2CO_3 (1.68 g, 12 mmol, 0.92 equiv), KI (10 mg, 60 μmol) and 1,3-dibromopropane (1.62 mL, 16 mmol, 1.30 equiv) in acetone (10 mL) was heated to reflux for 16 h.

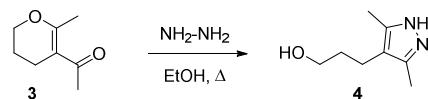
The inorganic salts were removed by filtration and the filtrate was concentrated under reduced pressure and the residue was purified by fractional distillation *in vacuo*. Boiling point of compound **3** was 55-58 °C/0.64-0.66 mbar. Yield: 850 mg, 6.06 mmol, 47%. ¹H NMR (400 MHz, CDCl₃, δ, ppm): 4.04 (t, *J* = 5.2 Hz, 2H), 2.40 – 2.36 (m, 2H), 2.22 (s, 3H), 2.21 (t, *J* = 1.4 Hz, 3H), 1.93 – 1.87 (m, 2H). HRMS calculated for C₈H₁₃O₂ (M+H)⁺ 141.0910, found 141.0913.

Scheme S4: Synthesis of compound **3**.



Synthesis of 3-(3,5-dimethyl-1H-pyrazol-4-yl)propan-1-ol (4) (Scheme S5). A solution of compound **3** (815 mg, 5.81 mmol, 1 equiv) of hydrazine monohydrate (64-65%, NH₂NH₂, 500 μL, 6.70 mmol, 1.15 equiv) in 2.5 mL EtOH was heated to reflux for 2 h. The solution was concentrated under reduced pressure and the residue was purified by column chromatography using 5% MeOH in dichloromethane (DCM) as eluent to obtain compound **4** as white crystals. Yield: 770 mg, 5 mmol, 86%. ¹H NMR (400 MHz, CD₃OD, δ, ppm): 3.53 (t, *J* = 6.6 Hz, 2H), 2.44 (t, *J* = 7.4 Hz, 2H), 2.16 (s, 6H), 1.71 – 1.62 (m, 2H). HRMS calculated for C₈H₁₅N₂O (M+H)⁺ 155.1179, found 155.1176.

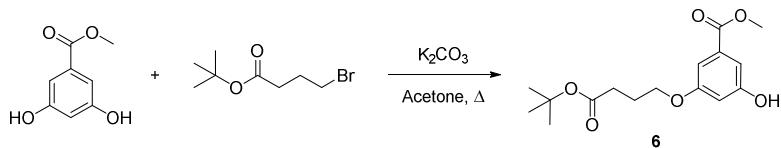
Scheme S5: Synthesis of compound **4**.



*Synthesis of Methyl 3-(4-(*tert*-butoxy)-4-oxobutoxy)-5-hydroxybenzoate (6) (Scheme S6).* K₂CO₃ (1.26 g, 9.16 mmol, 1.10 equiv) and *tert*-butyl 4-bromobutanoate (2 mL, 11.28 mmol, 1.35 equiv) were added to a solution of methyl-3,5-dihydroxybenzoate (1.40 g, 8.33 mmol, 1 equiv) in 20 mL acetone. The suspension was heated to reflux for 18 h. The inorganic salt was removed by filtration and the filtrate was concentrated under reduced pressure. The residue was purified by column chromatography with 12.5% ethyl acetate in hexane as eluent to obtain compound **6** as white crystals.

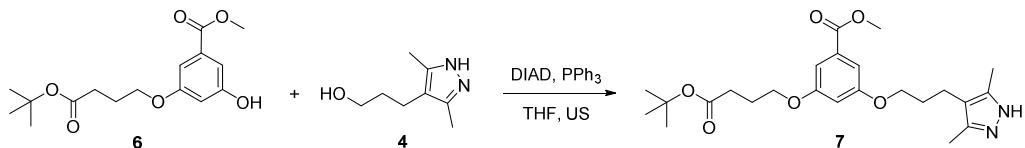
Yield: 962 mg, 3.10 mmol, 37%. ^1H NMR (400 MHz, CD_3OD , δ , ppm): 7.02 (d, J = 2.2 Hz, 2H), 6.57 (t, J = 2.2 Hz, 1H), 3.99 (t, J = 6.2 Hz, 2H), 3.87 (s, 3H), 2.41 (t, J = 7.2 Hz, 2H), 2.06 – 1.99 (m, 2H), 1.45 (s, 9H). HRMS calculated for $\text{C}_{15}\text{H}_{20}\text{N}_2\text{O}_4$ ($\text{M}+\text{H}$) $^+$ 291.1339, found 291.1336.

Scheme S6: Synthesis of compound **6**.



*Synthesis of Methyl 3-(4-(tert-butoxy)-4-oxobutoxy)-5-(3-(3,5-dimethyl-1*H*-pyrazol-4-yl)propoxy)benzoate (7) (Scheme S7).* Compound **6** (310.35 mg, 1 mmol, 1 equiv), compound **4** (161.92 mg, 1.05 mmol, 1.05 equiv), triphenylphosphine (275.40 mg, 1.05 mmol, 1.05 equiv) and 700 μL tetrahydrofuran were added to a 5 mL pear flask. The reactions vessel was placed in a 42 KHz ultrasonic bath and sonicated for 2 min. Then diisopropyl azodicarboxylate (206.70 μL , 1.05 mmol, 1.05 equiv) was added dropwise over the course of 2 min and sonicated for 15 min. The reaction mixture was concentrated under reduced pressure. The residue was purified by column chromatography using a gradient of 100% DCM to 0.5% MeOH in DCM to obtain compound **7** as white crystals. Yield: 395 mg, 0.88 mmol, 88%. ^1H NMR (400 MHz, CDCl_3 , δ , ppm) 7.14 (d, J = 2.2 Hz, 2H), 6.61 (t, J = 2.2 Hz, 1H), 4.00 (t, J = 6.2 Hz, 2H), 3.90 (t, J = 6.0 Hz, 2H), 3.88 (s, 3H), 2.54 (t, J = 7.2 Hz, 2H), 2.41 (t, J = 7.2 Hz, 2H), 2.17 (s, 6H), 2.09 – 2.01 (m, 2H), 1.96 – 1.87 (m, 2H), 1.44 (s, 9H). HRMS calculated for $\text{C}_{24}\text{H}_{35}\text{N}_2\text{O}_6$ ($\text{M}+\text{H}$) $^+$ 447.2490, found 447.2488.

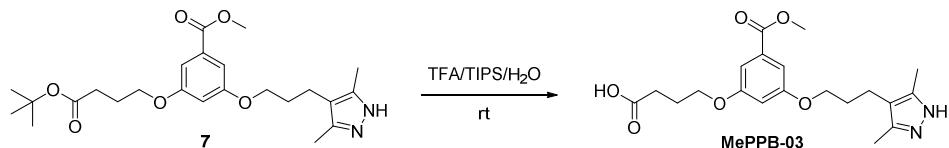
Scheme S7: Synthesis of compound **7**.



*Synthesis of 4-(3-(3,5-dimethyl-1*H*-pyrazol-4-yl)propoxy)-5-(methoxycarbonylphenoxy)butanoic acid (MePPB-03) (Scheme S8).* Cleavage of *tert*-butyl

ester of compound **7** (250 mg, 0.56 mmol) was achieved in a mixture of trifluoroacetic acid (TFA), triisopropylsilyl ether (TIPS) and H₂O in a ratio of 95:2.5:2.5 (800 μ L) for 1h at room temperature. After the evaporation of the solvents under reduced pressure column chromatography was carried out using a gradient of 100% DCM to 3% MeOH in DCM to obtain compound PPB-03 as white crystals. Yield: 150.71 mg, 386 μ mol, 69%. ¹H NMR (400 MHz, CD₃OD, δ , ppm) 7.15 (dd, J = 2.2, 1.2 Hz, 1H), 7.11 (dd, J = 2.2, 1.2 Hz, 1H), 6.71 (t, J = 2.2 Hz, 1H), 4.04 (t, J = 6.2 Hz, 2H), 3.97 (t, J = 6.0 Hz, 2H), 3.88 (s, 3H), 2.69 (t, J = 7.2 Hz, 2H), 2.49 (t, J = 7.2 Hz, 2H), 2.29 (s, 6H), 2.11 – 2.03 (m, 2H), 2.02 – 1.95 (m, 2H). HRMS calculated for C₂₀H₂₇N₂O₆ (M+H)⁺ 391.1864, found 391.1860.

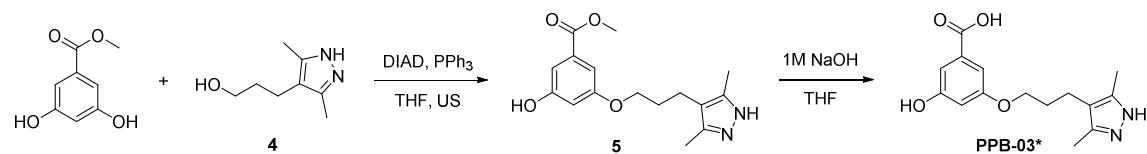
Scheme S8: Synthesis of compound MePPB-03.



Organic Synthesis of Synthesis of 3-(3-(3,5-dimethyl-1H-pyrazol-4-yl)propoxy)-5-hydroxybenzoic acid, a truncated version of PPB-03 (PPB-03*) (Scheme S9). Methyl-3,5-dihydroxybenzoate (168.15 mg, 1 mmol, 1 equiv), 3-(3,5-dimethyl-1H-pyrazol-4-yl)propan-1-ol (**4**) (161.92 mg, 1.05 mmol, 1.05 equiv), triphenylphosphine (275.40 mg, 1.05 mmol, 1.05 equiv) and 700 μ L tetrahydrofuran were added to a 5 mL pear-flask. The reactions vessel was placed in a 42 KHz ultrasonic bath and sonicated for 2 min. Then, diisopropyl azodicarboxylate (DIAD, 206.70 μ L, 1.05 mmol, 1.05 equiv) was added dropwise over the course of 5 min and sonicated for 45 more min. The reaction mixture was concentrated under reduced pressure. The residue was purified by column chromatography with a gradient of 100% DCM to 2% MeOH in DCM to obtain compound **5** (152 mg, 0.50 mmol) as white crystals. Compound **5** (152 mg, 0.50 mmol) was dissolved in 400 μ L tetrahydrofuran followed by addition of 2 mL 1 M NaOH. The solution was stirred at room temperature for 4 h. Then, the solution was neutralized by 1 M HCl and the precipitated crystals of PPB-03* were filtered and washed using acetonitrile. Yield: 133 mg, 0.46 mmol, 46%. ¹H NMR (400 MHz, CD₃OD, δ , ppm) 6.86 (dd, J = 2.2, 1.4 Hz, 1H), 6.67 (dd, J = 2.2, 1.4 Hz, 1H), 6.30 (t, J = 2.2

Hz, 1H), 3.84 (t, J = 6.0 Hz, 2H), 2.55 (t, J = 7.2 Hz, 2H), 2.13 (s, 6H), 1.92 – 1.82 (m, 2H). HRMS calculated for $C_{15}H_{19}N_2O_4$ ($M+H$)⁺ 291.1339, found 291.1336.

Scheme S9: Synthesis of compound PPB-03*, a truncated version of PPB-03.



2. In Vitro Properties of the Radioligands

A summary of the in vitro properties is given in Table S1. These values are presented in Figure 2 and Figure 3 of the main manuscript.

Table S1. Summary of the in Vitro Properties of the ^{177}Lu -Labeled Ligands

	^{177}Lu - DOTA- PPB-01	^{177}Lu - DOTA- PPB-02	^{177}Lu - DOTA- PPB-03	^{177}Lu - DOTA
HPLC retention times [min]	11.8	11.3	10.1	0.5**
LogD value	-3.15 ± 0.04	-4.01 ± 0.03	<-5	<-5
Plasma protein binding [%]	93 ± 1.5	70 ± 0.6	94 ± 0.6	<1
HSA binding [%]	95 ± 0.6	83 ± 0.6	57 ± 0.6	<1
Human $\alpha 1\text{-AGP}$ [%]	39 ± 9.6	13 ± 4.5	4.0 ± 1.1	<1
Human Transthyretin* [%]	9.0 ± 1.0	7.0 ± 2.1	50 ± 0.6	<1

* also referred to as prealbumin; ** TLC R_f value

3. Stability of the Radioligands

Experimental

The three compounds DOTA-PPB-01, DOTA-PPB-02, DOTA-PPB-03 were labeled at a specific activity of 50 MBq/nmol for testing the stability in PBS pH 7.4 and human plasma. The radiochemical purity was determined immediately after production (T = 0) using HPLC. Dilutions of the labeling solution of each compound were prepared at low activity concentration (~10 MBq/250 µL) using PBS pH 7.4 with and without the addition of ascorbic acid (~120 µg). In addition, the stability of the radioligands was checked at higher activity concentrations (250 MBq/500 µL) with the addition of a higher amount of ascorbic acid (3 mg). In the latter case, it was necessary to adjust the pH by addition of a solution of sodium acetate (0.5 M, pH 8, 150 µL) to obtain a value of 7.0-7.5. The integrity of the radioligands was determined after incubation periods of 4 h, 24 h and 48 h.

For the determination of plasma stability, the radioligands were diluted to 30 MBq/500 µL using PBS pH 7.4. An aliquot of the prepared dilutions (50 µL, ~3 MBq) were incubated in human plasma (Foundation Blood Donation SRK Aargau-Solothurn, Switzerland, ~250 µL) at 37 °C. The compounds' integrity was determined after 4, 24 and 48 h. For this purpose aliquots of the plasma (50 µL) were treated with three-fold volume of methanol to precipitate plasma proteins. After centrifugation the supernatants were filtered and used for analysis using HPLC.

The HPLC chromatograms were analyzed by determination of the peak area of the radioligand and set into relation to the area of the radioligand observed at T = 0 which was set to 100%.

Results and Conclusion

Preparation and Stability ^{177}Lu -Labeled Ligands. The radiochemical purity of ^{177}Lu -DOTA-PPB-01, ^{177}Lu -DOTA-PPB-02 and ^{177}Lu -DOTA-PPB-03, labeled at a specific activity of 50 MBq/nmol, was ≥97%. The HPLC retention times were similar (~10 min), for all three radioligands (Table 2). The radiochemical purity of ^{177}Lu -DOTA, labeled at a specific activity of 50 MBq/nmol, was ≥97%. Stability experiments revealed that ^{177}Lu -DOTA-PPB-01 in PBS pH 7.4 (10 MBq/250 µL) was not sufficiently stable as only 88% intact compound were found after 4 h and this amount dropped

further to 32% intact compound after 48 h. The addition of ascorbic acid increased the stability of ^{177}Lu -DOTA-PPB-01 significantly demonstrated by 97% completely intact after 4 h and 88% after 48 h. ^{177}Lu -DOTA-PPB-02 (10 MBq/250 μL) showed a similar behavior. Without ascorbic acid the product peak quickly decreased to 94% after 4 h and dropped further to 42% after 48 h. Stability was increased by addition of ascorbic acid resulting in 97% intact compound after 4 h and 87% after 48 h. ^{177}Lu -DOTA-PPB-03 (10 MBq/250 μL) revealed to be more stable with >98% intact product after 4 h and still >75% after 48 h. The addition of ascorbic acid improved the stability allowing to maintain the compound completely intact (>98%) over the whole time of investigation (Figure S2).

With the addition of ascorbic acid (3 mg) all radioligands were stable even when diluted at high activity concentration (250 MBq/500 μL). ^{177}Lu -DOTA-PPB-01 and ^{177}Lu -DOTA-PPB-02 were almost completely intact (>97%) after 4 h and showed only slight degradation after 24 h (>90% and >92% intact radioligand, respectively). Both radioligands remained relatively stable showing ~88% intact product at 48 h. Due to the high stability shown for ^{177}Lu -DOTA-PPB-03 at low activity concentrations, this compound was also evaluated at high activity concentration (250 MBq/500 μL) without addition of ascorbic acid. It was shown that this radioligand remained largely intact (~91%) after 4 h, but after 24 h only ~55% of intact radioligand was determined, however, when ascorbic acid was added the radioligand remained intact (>98%) over 48 h. All the three radioligands were stable (>95%) in human plasma over 24 h and even after 48 h, a high fraction of these radioligands were still intact (>93%).

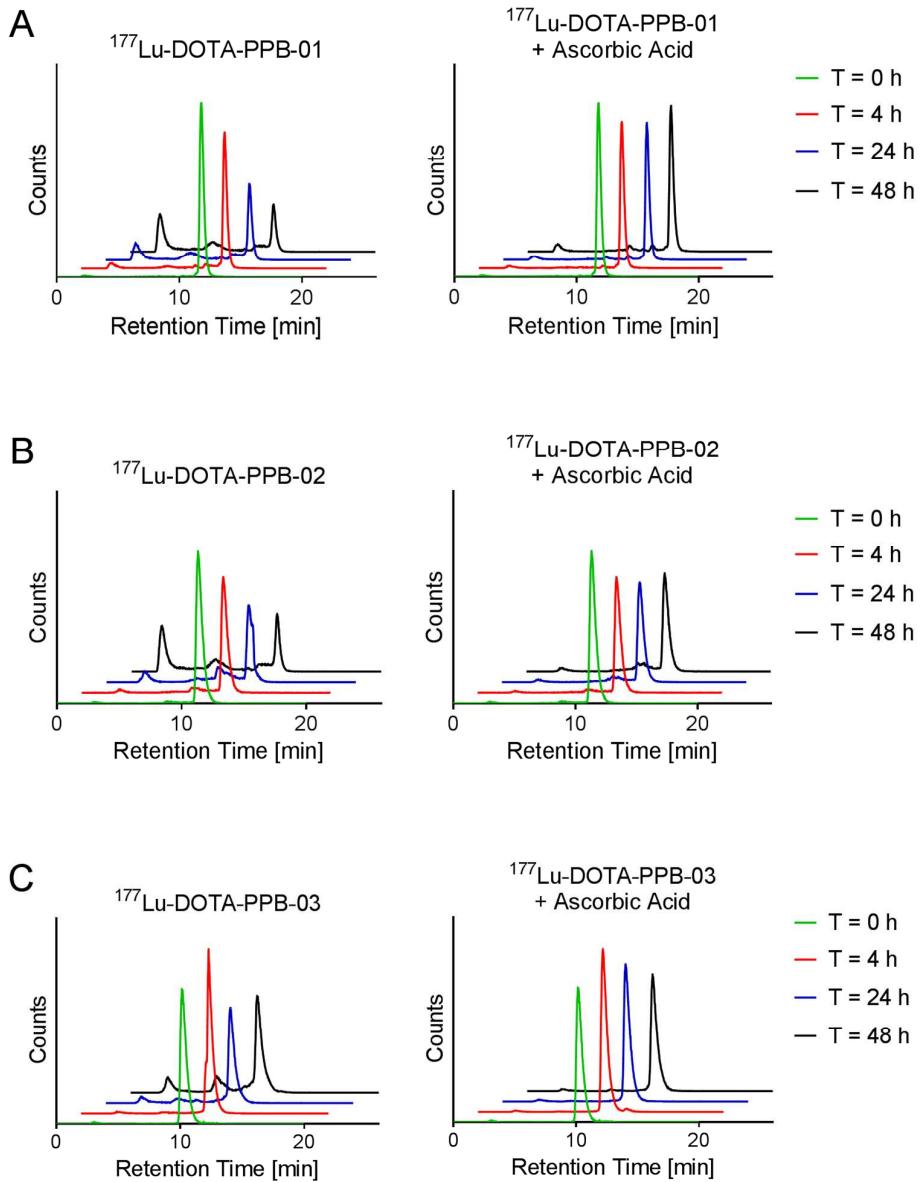


Figure S2. Results of the stability experiments performed with (A) ^{177}Lu -DOTA-PPB-01, (B) ^{177}Lu -DOTA-PPB-02 and (C) ^{177}Lu -DOTA-PPB-03. The stability was evaluated for the three radioligands diluted in PBS (10 MBq/250 μL), with and without the addition of the scavenger ascorbic acid, after incubation of up to 48 h.