## General Information

Analytical and preparative high-performance liquid chromatography (HPLC) was performed using Shimadzu gradient systems (Neufahrn, Germany) equipped with an SPD-20A UV/Vis detector. The columns for analytical (MultoKrom 100C18, 150×4.6 mm, 5 μm), radio-analytical (Multospher 100RP18, 125×4.6 mm, 5 μm) and preparative (MultoKrom 100C18, 250×20 mm, 5 μm) HPLC were purchased from CS Chromatographie Service (Langerwehe, Germany). Eluents for all HPLC operations were water (solvent A) and acetonitrile with 2 vol.% water (solvent B), both containing 0.1 vol.% trifluoroacetic acid (TFA). Radioactivity was detected via a HERM LB 500 NaI detector (Berthold Technologies, Bad Wildbad, Germany). Radio-thin layer chromatography (TLC) was carried out with a Scan-RAM detector (LabLogic Systems, Sheffield, United Kingdom). Electrospray ionization-mass spectra were acquired on an expression[L] CMS (Advion, Harlow, UK).

## Synthesis of PSMA Ligands

The uncomplexed radiohybrid ligands rhPSMA-7.1, -7.2, -7.3 and -7.4 were prepared by applying a mixed solid phase/solution phase synthetic strategy, according to the literature protocols (*1*). rhPSMA-10.1 and -10.2 were obtained in analogy to the rhPSMA-7 isomers, by substitution of the DOTA-GA chelator with DOTA. PSMA I&T was prepared according to the published procedure (*2*) and PSMA-617 was purchased from MedChemExpress LLC (Monmouth Junction, USA).

For complexation with non-radioactive lutetium for *in vitro* studies, a 2 mM solution of the PSMA inhibitor (1.0 eq.) in DMSO was combined with a 20 mM aqueous solution of $LuCl_3$ (2.5 eq.) and heated to 95°C for 30 min. Analytical data of the Lu-chelated PSMA ligands are summarized in the supporting information.

## Analytical data of Lu-complexed PSMA inhibitors:

**Lu-rhPSMA-7.1:** RP-HPLC (10 to 70% B in 15 min): $t_R$ = 9.7 min, K' = 3.85. Calculated monoisotopic mass ($C_{63}H_{96}FLuN_{12}O_{25}Si$): 1642.6; found: m/z = 1643.5 $[M+H]^+$, 822.5 $[M+2H]^{2+}$.

**Lu-rhPSMA-7.2:** RP-HPLC (10 to 70% B in 15 min): $t_R$ = 9.4 min, K' = 3.70. Calculated monoisotopic mass ($C_{63}H_{96}FLuN_{12}O_{25}Si$): 1642.6; found: m/z = 1642.9 $[M+H]^+$, 822.0 $[M+2H]^{2+}$.

**Lu-rhPSMA-7.3:** RP-HPLC (10 to 70% B in 15 min)*: $t_R$ = 9.6 min,* K' = 3.80*. Calculated monoisotopic mass ($C_{63}H_{96}FLuN_{12}O_{25}Si$): 1642.6; found: m/z = 1643.4 $[M+H]^+$, 822.3 $[M+2H]^{2+}$.

**Lu-rhPSMA-7.4:** RP-HPLC (10 to 70% B in 15 min): $t_R$ = 9.6 min,* K' = 3.80*. Calculated monoisotopic mass ($C_{63}H_{96}FLuN_{12}O_{25}Si$): 1642.6; found: m/z = 1643.0 $[M+H]^+$, 822.3 $[M+2H]^{2+}$.

**Lu-rhPSMA-10.1:** RP-HPLC (10 to 70% B in 15 min): $t_R$ = 9.9 min, K' = 3.95. Calculated monoisotopic mass ($C_{60}H_{92}FLuN_{12}O_{23}Si$): 1570.6; found: m/z = 1571.8 $[M+H]^+$, 786.2 $[M+2H]^{2+}$.

**Lu-rhPSMA-10.2:** RP-HPLC (10 to 70% B in 15 min): $t_R$ = 9.6 min, K' = 3.80. Calculated monoisotopic mass ($C_{60}H_{92}FLuN_{12}O_{23}Si$): 1570.6; found: m/z = 1571.9 $[M+H]^+$, 786.6 $[M+2H]^{2+}$.

**Lu-PSMA-I&T:** RP-HPLC (10 to 70% B in 15 min): $t_R$ = 7.2 min, K' = 3.32. Calculated monoisotopic mass ($C_{63}H_{89}ILuN_{11}O_{23}$): 1669.5; found: m/z = 1670.5 $[M+H]^+$, 1113.8 $[2M+3H]^{3+}$.

**Lu-PSMA-617:** RP-HPLC (10 to 70% B in 15 min): $t_R$ = 6.5 min, K' = 2.82. Calculated monoisotopic mass ($C_{49}H_{68}LuN_9O_{16}$): 1213.4; found: m/z = 1213.6 $[M+H]^+$, 607.5 $[M+2H]^{2+}$.

**Supplemental Table 1:** Radiochemical purity (RCP) of ^{177}Lu-labeled rhPSMA isomers, determined by radio-TLC before lipophilicity studies, using either the citrate buffer system (0.1 M sodium citrate buffer on iTLC-SG chromatography paper) or acetate buffer system (1.0 M $NH_4OAc$/DMF buffer (1/1; v/v) on TLC Silica gel 60 F254 plates).

| [177Lu]Lu- | citrate-TLC RCP | acetate-TLC RCP |
|---|---|---|
| rhPSMA-7.1 | 99.5% | 99.7% |
| rhPSMA-7.2 | 99.8% | 99.8% |
| rhPSMA-7.3 | 99.8% | 99.9% |
| rhPSMA-7.4 | 99.8% | 99.8% |
| rhPSMA-10.1 | 99.8% | 99.8% |
| rhPSMA-10.2 | 99.7% | 99.7% |

**Supplemental Table 2:** Binding affinities (IC$_{50}$ [nM], 1 h, 4°C) of Lu-rhPSMA-7.1 to -7.4 (n=3), Lu-rhPSMA-10.1, -10.2 (n=3) and the references Lu-PSMA-617 and Lu-PSMA-I&T (n=3); PSMA-mediated internalization of [$^{177}$Lu]Lu-rhPSMA-7.1 to -7.4 (n=3), [$^{177}$Lu]Lu-rhPSMA-10.1, -10.2 (n=3) and the references [$^{177}$Lu]Lu-PSMA-617 and [$^{177}$Lu]Lu-PSMA I&T (n=3) by LNCaP cells (1 h, 37°C) as a percentage of the reference ligand ([$^{125}$I]IBA)KuE); Lipophilicity of [$^{177}$Lu]Lu-rhPSMA-7.1 to -7.4 (n=6), [$^{177}$Lu]Lu-rhPSMA-10.1, -10.2 (n=6) and the references [$^{177}$Lu]Lu-PSMA-617 and [$^{177}$Lu]Lu-PSMA I&T (n=6), expressed as partition coefficient (log $D_{7.4}$) using the *n*-octanol/PBS (pH 7.4) distribution system ; Apparent molecular weight (AMW) of [$^{177}$Lu]Lu-rhPSMA-7.1 to -7.4, [$^{177}$Lu]Lu-rhPSMA-10.1, -10.2 and the references [$^{177}$Lu]Lu-PSMA-617 and [$^{177}$Lu]Lu-PSMA I&T, as determined by human serum albumin mediated size exclusion chromatography (AMSEC).

| Compound | [$^{177}$Lu]Lu-rhPSMA-7.1 | [$^{177}$Lu]Lu-rhPSMA-7.2 | [$^{177}$Lu]Lu-rhPSMA-7.3 | [$^{177}$Lu]Lu-rhPSMA-7.4 | [$^{177}$Lu]Lu-rhPSMA- | [$^{177}$Lu]Lu-rhPSMA- | [$^{177}$Lu]Lu-PSMA-617 | [$^{177}$Lu]Lu-PSMA I&T |
|---|---|---|---|---|---|---|---|---|
| IC$_{50}$ [nM] | 3.11 ± 0.64 | 2.88 ± 1.06 | 3.29 ± 1.00 | 3.06 ± 1.51 | 2.76 ± 0.51 | 3.61 ± 0.59 | 3.27 ± 0.19 | 4.17 ± 0.78 |
| Internalization [%IBA-KuE] | 137.4 ± 5.8 | 197.3 ± 15.0 | 184.4 ± 11.8 | 190.4 ± 10.5 | 177.4 ± 14.6 | 205.6 ± 8.3 | 203.2 ± 10.1 | 145.4 ± 13.8 |
| log$D_{7.4}$ | -4.27 ± 0.24 | -4.25 ± 0.29 | -4.12 ± 0.11 | -4.10 ± 0.14 | -3.78 ± 0.06 | -3.83 ± 0.10 | -4.1 ± 0.1 | -4.1 ± 0.1 |
| MW$_{app}$ [kDa] | 26.3 | 31.7 | 30.4 | 35.7 | 25.1 | 21.8 | 13.7 | 5.3 |

**Supplemental Table 3:** Biodistribution of [$^{177}$Lu]Lu-rhPSMA-7.1 to -7.4 at 24 h p.i. in male LNCaP tumor-bearing SCID mice. Data are expressed as a percentage of the injected dose per gram (% ID/g), mean ± standard deviation (SD; n=4).

| uptake in % ID/g | [$^{177}$Lu]Lu-rhPSMA-7.1 24 h p.i., n = 4 | | [$^{177}$Lu]Lu-rhPSMA-7.2 24 h p.i., n = 4 | | [$^{177}$Lu]Lu-rhPSMA-7.3 24 h p.i., n = 4 | | [$^{177}$Lu]Lu-rhPSMA-7.4 24 h p.i., n = 4 | |
|---|---|---|---|---|---|---|---|---|
| | mean | SD | mean | SD | mean | SD | mean | SD |
| **blood** | **0.0023** | **0.0006** | **0.0090** | **0.0015** | **0.0032** | **0.0007** | **0.0052** | **0.0026** |
| heart | 0.036 | 0.009 | 0.032 | 0.005 | 0.031 | 0.009 | 0.032 | 0.006 |
| lung | 0.050 | 0.006 | 0.049 | 0.009 | 0.049 | 0.011 | 0.056 | 0.014 |
| **liver** | **0.28** | **0.06** | **0.24** | **0.06** | **0.22** | **0.04** | **0.16** | **0.03** |
| spleen | 0.24 | 0.04 | 0.79 | 0.13 | 0.73 | 0.23 | 0.72 | 0.29 |
| pancreas | 0.019 | 0.003 | 0.076 | 0.071 | 0.025 | 0.005 | 0.027 | 0.006 |
| stomach | 0.071 | 0.018 | 0.246 | 0.236 | 0.059 | 0.028 | 0.068 | 0.029 |
| intestine | 0.15 | 0.05 | 0.34 | 0.31 | 0.13 | 0.08 | 0.15 | 0.07 |
| **kidney** | **4.10** | **0.81** | **19.04** | **4.45** | **9.82** | **2.74** | **15.66** | **8.92** |
| adrenals | 0.12 | 0.04 | 0.61 | 0.13 | 0.56 | 0.22 | 0.34 | 0.18 |
| muscle | 0.011 | 0.001 | 0.010 | 0.002 | 0.009 | 0.003 | 0.010 | 0.003 |
| bone | 0.030 | 0.009 | 0.034 | 0.008 | 0.048 | 0.010 | 0.023 | 0.009 |
| **tumor** | **12.40** | **1.27** | **12.73** | **2.76** | **11.63** | **2.24** | **12.56** | **3.52** |
| parotid gl. | 0.083 | 0.020 | 0.114 | 0.030 | 0.131 | 0.042 | 0.111 | 0.057 |
| submand. gl. | 0.051 | 0.004 | 0.072 | 0.005 | 0.056 | 0.010 | 0.075 | 0.019 |

**Supplemental Table 4:** Biodistribution of [177Lu]Lu-rhPSMA-10.1, -10.2 and the references [177Lu]Lu-PSMA-617 and [177Lu]Lu-PSMA I&T at 24 h p.i. in male LNCaP tumor-bearing SCID mice. Data are expressed as a percentage of the injected dose per gram (% ID/g), mean ± standard deviation (SD; n=4-5). Values of [177Lu]Lu-PSMA I&T were taken from a previously published study by our group (3). N.d. not determined.

| uptake in % ID/g | [177Lu]Lu-rhPSMA-10.1 24 h p.i., n = 5 | | [177Lu]Lu-rhPSMA-10.2 24 h p.i., n = 4 | | [177Lu]Lu-PSMA-617 24 h p.i., n = 4 | | [177Lu]Lu-PSMA-I&T 24 h p.i., n = 4 | |
|---|---|---|---|---|---|---|---|---|
| | mean | SD | mean | SD | mean | SD | mean | SD |
| **blood** | **0.0009** | **0.0001** | **0.0022** | **0.0008** | **0.0056** | **0.0026** | **0.0118** | **0.0064** |
| heart | 0.017 | 0.001 | 0.020 | 0.004 | 0.012 | 0.004 | 0.049 | 0.034 |
| lung | 0.032 | 0.004 | 0.036 | 0.007 | 0.039 | 0.013 | 0.158 | 0.029 |
| **liver** | **0.18** | **0.06** | **0.15** | **0.05** | **0.12** | **0.06** | **0.05** | **0.01** |
| spleen | 0.17 | 0.03 | 0.53 | 0.26 | 0.08 | 0.01 | 1.94 | 1.01 |
| pancreas | 0.013 | 0.002 | 0.021 | 0.002 | 0.011 | 0.004 | 0.048 | 0.029 |
| stomach | 0.056 | 0.014 | 0.104 | 0.069 | 0.020 | 0.003 | 0.048 | 0.021 |
| intestine | 0.11 | 0.05 | 0.24 | 0.13 | 0.12 | 0.08 | 0.12 | 0.06 |
| **kidney** | **1.97** | **0.78** | **8.09** | **1.68** | **1.44** | **0.42** | **34.66** | **17.20** |
| adrenals | 0.06 | 0.04 | 0.41 | 0.17 | 0.19 | 0.07 | 1.06 | 0.24 |
| muscle | 0.003 | 0.002 | 0.007 | 0.002 | 0.009 | 0.002 | 0.010 | 0.004 |
| bone | 0.022 | 0.008 | 0.020 | 0.008 | 0.027 | 0.017 | 0.014 | 0.004 |
| **tumor** | **9.82** | **0.30** | **10.45** | **3.25** | **7.46** | **0.90** | **4.06** | **1.12** |
| parotid gl. | 0.041 | 0.009 | 0.100 | 0.017 | n.d. | n.d. | n.d. | n.d. |
| submand. gl. | 0.037 | 0.007 | 0.046 | 0.009 | n.d. | n.d. | n.d. | n.d. |

**Supplemental Table 5:** Tumor-to-organ ratios of [¹⁷⁷Lu]Lu-rhPSMA-7.1 to -7.4 at 24 h p.i. in male LNCaP tumor-bearing SCID mice. Mean ± standard deviation (SD; n=4). Tumor-to-organ ratios were calculated from individual mice and mean values were determined.

| tumor-to-organ ratio | [¹⁷⁷Lu]Lu-rhPSMA-7.1 24 h p.i., n = 4 | | [¹⁷⁷Lu]Lu-rhPSMA-7.2 24 h p.i., n = 4 | | [¹⁷⁷Lu]Lu-rhPSMA-7.3 24 h p.i., n = 4 | | [¹⁷⁷Lu]Lu-rhPSMA-7.4 24 h p.i., n = 4 | |
|---|---|---|---|---|---|---|---|---|
| | **mean** | **SD** | **mean** | **SD** | **mean** | **SD** | **mean** | **SD** |
| **blood** | **5971** | **2358** | **1460** | **495** | **3843** | **1145** | **2570** | **820** |
| heart | 368 | 122 | 409 | 125 | 394 | 82 | 387 | 81 |
| lung | 252 | 53 | 275 | 113 | 239 | 24 | 234 | 87 |
| **liver** | **47** | **13** | **54** | **12** | **54** | **2** | **78** | **13** |
| spleen | 54 | 13 | 16 | 3 | 17 | 3 | 19 | 4 |
| pancreas | 671 | 211 | 285 | 170 | 487 | 133 | 489 | 180 |
| stomach | 191 | 70 | 187 | 210 | 262 | 177 | 228 | 135 |
| intestine | 95 | 40 | 150 | 211 | 129 | 86 | 104 | 55 |
| **kidney** | **3.2** | **0.9** | **0.7** | **0.2** | **1.2** | **0.3** | **1.0** | **0.5** |
| adrenals | 119 | 54 | 21 | 1 | 23 | 7 | 43 | 14 |
| muscle | 1167 | 214 | 1453 | 623 | 1454 | 601 | 1269 | 92 |
| bone | 453 | 190 | 384 | 66 | 264 | 111 | 616 | 309 |
| parotid gl. | 163 | 69 | 112 | 14 | 93 | 21 | 99 | 13 |
| submand. gl. | 244 | 43 | 177 | 40 | 207 | 27 | 169 | 33 |

**Supplemental Table 6:** Tumor-to-organ ratios [$^{177}$Lu]Lu-rhPSMA-10.1, -10.2 and the references [$^{177}$Lu]Lu-PSMA-617 and [$^{177}$Lu]Lu-PSMA I&T at 24 h p.i. in male LNCaP tumor-bearing SCID mice. Mean ± standard deviation (SD; n=4-5). Values of [$^{177}$Lu]PSMA I&T were taken from a previously published study by our group (*3*). Tumor-to-organ ratios were calculated from individual mice and mean values were determined.

| tumor-to-organ ratio | [$^{177}$Lu]Lu-rhPSMA-10.1 24 h p.i., n = 5 | | [$^{177}$Lu]Lu-rhPSMA-10.2 24 h p.i., n = 4 | | [$^{177}$Lu]Lu-PSMA-617 24 h p.i., n = 4 | | [$^{177}$Lu]Lu-PSMA-I&T 24 h p.i., n = 4 | |
|---|---|---|---|---|---|---|---|---|
| | mean | SD | mean | SD | mean | SD | mean | SD |
| **blood** | **11498** | **1953** | **6125** | **4185** | **1424** | **455** | **408** | **209** |
| heart | 568 | 52 | 585 | 321 | 704 | 270 | 128 | 77 |
| lung | 313 | 33 | 295 | 100 | 210 | 74 | 26 | 9 |
| **liver** | **57** | **13** | **69** | **17** | **91** | **73** | **86** | **31** |
| spleen | 58 | 11 | 27 | 19 | 93 | 22 | 3.0 | 2.0 |
| pancreas | 774 | 189 | 511 | 198 | 799 | 293 | 144 | 115 |
| stomach | 187 | 47 | 221 | 231 | 376 | 65 | 105 | 60 |
| intestine | 115 | 75 | 111 | 162 | 90 | 50 | 49 | 33 |
| **kidney** | **5.7** | **2.0** | **1.4** | **0.6** | **5.9** | **3.0** | **0.15** | **0.08** |
| adrenals | 171 | 111 | 33 | 22 | 44 | 15 | 4.1 | 1.5 |
| muscle | 2441 | 373 | 1798 | 792 | 855 | 279 | 496 | 242 |
| bone | 595 | 462 | 589 | 280 | 472 | 378 | 315 | 144 |
| parotid gl. | 250 | 64 | 107 | 37 | n.d. | n.d. | n.d. | n.d. |
| submand. gl. | 275 | 49 | 223 | 45 | n.d. | n.d. | n.d. | n.d. |

mV
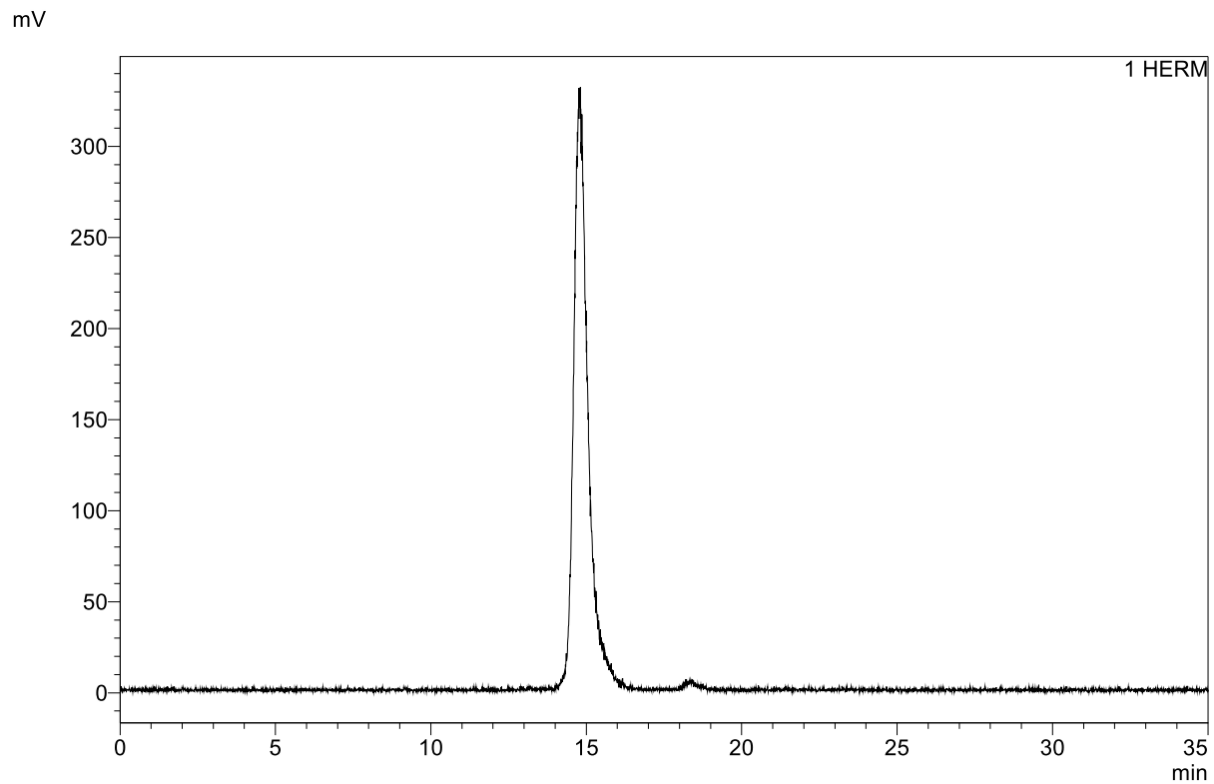


**Supplemental Figure 1:** Exemplary radio-chromatogram of [$^{177}$Lu]Lu-rhPSMA-7.3 in the AMSEC experiment. The retention time of 14.8 min measured in this particular experiment correlates with an AMW of 30.3 kDa (AMW of 30.4 ± 0.5 kDa (mean ± SD) for n = 10 independent measurements).
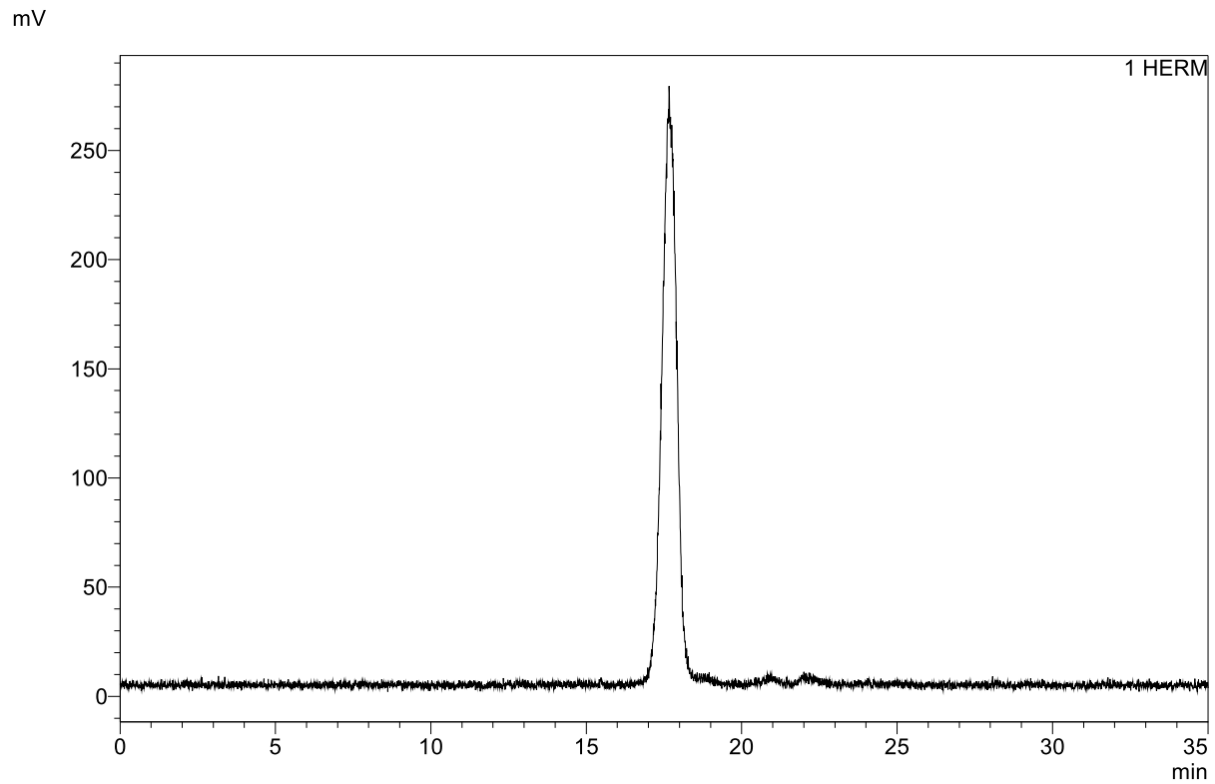
**Supplemental Figure 2:** Radio-chromatogram of [$^{177}$Lu]Lu-PSMA-617 in the AMSEC experiment. The retention time of 17.7 min measured in this particular experiment correlates with an AMW of 13.7 kDa.
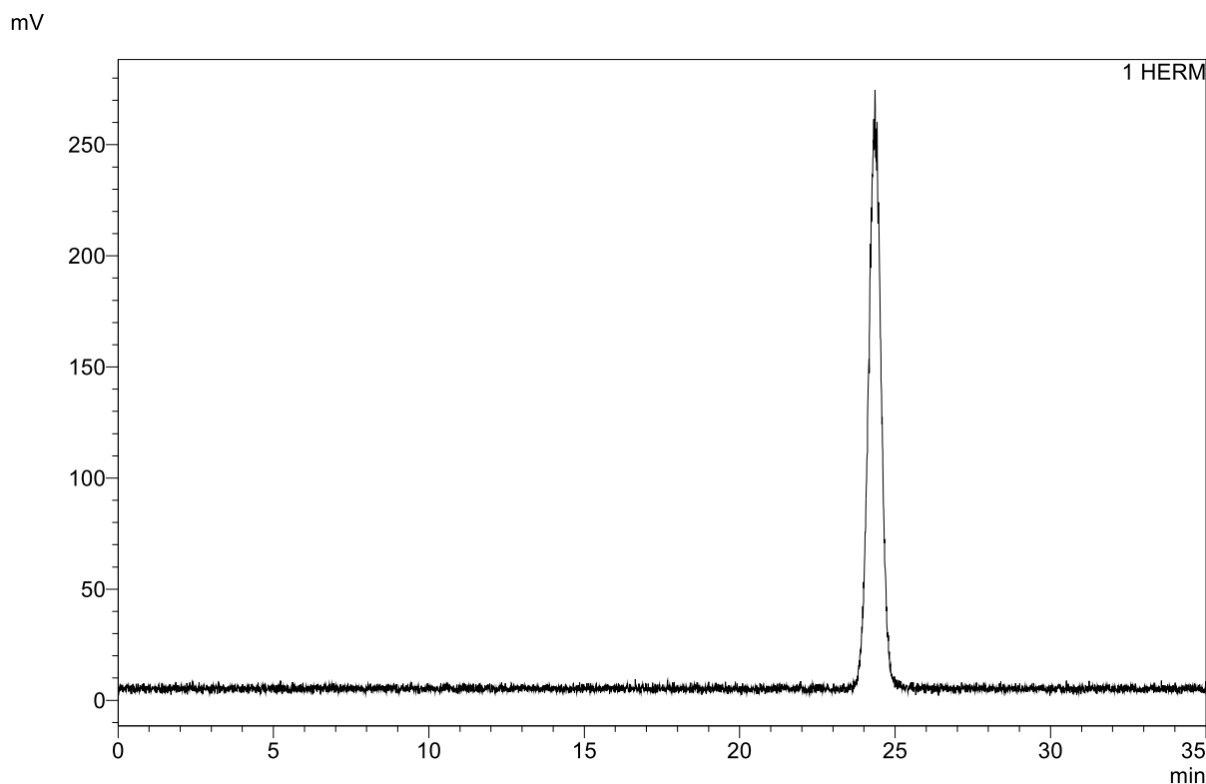
**Supplemental Figure 3:** Radio-chromatogram of [$^{18}$F]fluoride in the AMSEC experiment. Due to the absence of interaction of fluoride with HSA, [$^{18}$F]fluoride is eluted after 24.4 min, as expected for a small inorganic ion, and the corresponding AMW of 2.1 kDa lies below the fractionation range of the gel filtration column (70 – 3 kDa).

**References**

**1.**     Wurzer A, Parzinger M, Konrad M, et al. Preclinical comparison of four [(18)F, (nat)Ga]rhPSMA-7 isomers: influence of the stereoconfiguration on pharmacokinetics. *EJNMMI Res.* 2020;10:149.

**2.**     Weineisen M, Schottelius M, Simecek J, et al. 68Ga- and 177Lu-Labeled PSMA I&T: Optimization of a PSMA-Targeted Theranostic Concept and First Proof-of-Concept Human Studies. *J Nucl Med.* 2015;56:1169-1176.

**3.**     Wirtz M, Schmidt A, Schottelius M, et al. Synthesis and in vitro and in vivo evaluation of urea-based PSMA inhibitors with increased lipophilicity. *EJNMMI Res.* 2018;8:84.