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# 1 Validation of HPLC and TLC analytical methods to determine radiochemical 2 purity of <sup>99m</sup>Tc-cAbVCAM1-5, a new experimental radiotracer

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## 14 HIGHLIGHTS

- 15 • <sup>99m</sup>Tc-cAbVCAM1-5 is a new experimental radiotracer targeting a marker of inflammation  
16 found in vulnerable atheromatous plaques in cardiac diseases.
- 17 • In order to complete an investigational medicinal product dossier, a validation of the  
18 analytical method ~~New radiotracer requires validation of analytical method~~ to assess  
19 radiochemical purity by HPLC and TLC is required.
- 20 • Specificity, accuracy, repeatability and intermediate precision, linearity, robustness,  
21 quantification limit (LoQ), and range criteria were assessed for HPLC and TLC method.
- 22 • The results developed in this study show that the analytical method is particularly suitable  
23 for assessing the radiochemical purity of <sup>99m</sup>Tc-cAbVCAM1-5 in our laboratory.

24

## 25 ABSTRACT

26

27 Cardiovascular diseases, including fatal myocardial infarctions from atheromatous plaques, are the  
28 primary global mortality cause. Detecting stenotic atheromatous plaques is possible through  
29 coronary angiography, but vulnerable plaques with eccentric remodeling are undetectable with  
30 current diagnostic methods. Addressing this challenge, our group developed a radiopharmaceutical  
31 drug targeting Vascular Cell Adhesion Molecule 1 (VCAM-1), radiolabeled with technetium-99m.  
32 Given the absence of a monograph in the European Pharmacopoeia, and in order to draft the  
33 investigational medicinal product documentation, analytical methods had to be validated by High  
34 Performance Liquid Chromatography (HPLC) and Thin Layer Chromatography (TLC) to  
35 determine the radiochemical purity (RCP) of <sup>99m</sup>Tc-cAbVCAM1-5. This study therefore presents the  
36 results of the validation of analytical methods obtained in this context. The method validation  
37 followed the European Association of Nuclear Medicine (EANM) recommendations adapted from ICH  
38 Q2(R1), ensuring conformity with specificity, accuracy, repeatability and intermediate precision,  
39 linearity, robustness, quantification limit (LoQ), and range criteria. Regarding the results of  
40 specificity, both HPLC and TLC methods demonstrated excellent separation of <sup>99m</sup>Tc-cAbVCAM1-5  
41 from impurities <sup>99m</sup>TcO<sub>4</sub><sup>-</sup>. Accuracy results indicated recovery percentages within the range of 99.52  
42 to 101.40 % for the HPLC and 99.51 to 101.97 % for TLC, ensuring reliable measurements for each  
43 concentration of <sup>99m</sup>TcO<sub>4</sub><sup>-</sup>. Precision of the methods was validated by assessing repeatability and  
44 intermediate precision. Linearity was determined over the usual concentrations range and the  
45 correlation coefficient was greater than 0.99 for both methods. The limit of quantification was  
46 measured by diluting the <sup>99m</sup>TcO<sub>4</sub><sup>-</sup> to obtain a signal-to-noise ratio of around 10:1. Under these

47 conditions, we obtained an LOQ of 2.10 MBq/mL for HPLC and 2Mbq/mL for TLC. In conclusion, the  
48 analytical methods developed in this study comply with EANM recommendations. This therefore  
49 allows us to correctly assess the radiochemical purity of <sup>99m</sup>Tc-cAbVCAM1-5, a new radiotracer  
50 targeting inflammation in vulnerable plaques.  
51

52 Keywords: High performance liquid chromatograph, method validation, radiopharmaceuticals,  
53 radiochemical purity, single domain antibody, thin layer chromatography, ~~method validation,~~  
54 ~~radiopharmaceuticals, radiochemical purity, single domain antibody.~~

## 55 1. Introduction

56 Cardiovascular diseases, particularly coronary artery disease, are the leading cause of death  
57 worldwide[1]. Atherosclerosis represents a global health challenge and the management of this  
58 disease is essential to prevent the risk of acute cardiovascular events[2]. Atheromatous plaques are  
59 lesions developed within the arterial wall. When they occur in the coronary arteries, they can lead to  
60 the formation of occlusive thrombus, which are responsible for myocardial infarction. Stenotic  
61 atheromatous plaques can be detected by coronary angiography because they affect the artery  
62 lumen. However, this is not the case for vulnerable plaques with eccentric remodelling which are  
63 usually associated with intense inflammatory phenomena but do not affect the lumen of the artery.  
64 In practice, 60 to 70% of myocardial infarctions are caused by the rupture of vulnerable plaque.  
65 However, no current diagnostic technique can effectively detect this type of plaque[3,4]. In this  
66 context our group has developed an anti-VCAM-1 single domain antibody (sdAb) radiolabeled with  
67 technetium-99m targeting a marker of inflammation: the ~~V~~ascular ~~C~~ell ~~A~~dhesion ~~M~~olecule 1  
68 (VCAM-1) whose expression is restricted to inflamed plaque. This has been validated in preclinical  
69 studies, and it could allow a non-invasive diagnosis by imaging of vulnerable plaque[5,6]. In addition  
70 to its clinical innovation, this novel radiotracer is distinctive in its vector (sdAb). There are only  
71 a few sdAb in clinical nuclear imaging, with the majority of them being radiolabelled with gallium-68.  
72 The only sdAb radiolabelled with <sup>99m</sup>Tc using the tricarbonyl method is an anti PD-L1 sdAb intended  
73 for patients with non-small cell lung cancer[7]. In the case of radiopharmaceuticals, a wide range of  
74 tests must be implemented in order to assess the quality of the final product and ultimately to  
75 release production batches [8–11]. Radiochemical purity (RCP) is one of the most crucial assays,  
76 defined as the ratio (in percentage) between the radioactivity of the radionuclide as a  
77 radiopharmaceutical and the total radioactivity of that nuclide in the pharmaceutical preparation  
78 (radiopharmaceutical + impurities) [10,12]. It is important to ascertain the proportion of these  
79 radiochemical impurities that are likely to be present or to appear in a preparation, because they  
80 lead to additional irradiation of the patient and, in particular, to impaired imaging that can have an  
81 impact on the diagnosis. Most of the time, this RCP evaluation is performed using analytical  
82 separation techniques such as thin layer chromatography or liquid chromatography. Analytical  
83 methods must be validated before being used in routine quality control procedures. The aim of  
84 validation is to ensure that methods are suitable for their intended use[13]. In order to obtain the  
85 agreement of the French national agency for medicines and health products to carry out a clinical  
86 study evaluating <sup>99m</sup>Tc-cAbVCAM1-5 as a new radiotracer, ~~we needed to fill~~ we had to complete an  
87 ~~investigational medicinal product dossier (IMPD)~~. Part of this IMPD is intended for analytical  
88 ~~methods validation in order to determine the RCP by High Performance Liquid Chromatography~~  
89 ~~(HPLC) and Thin Layer Chromatography (TLC),~~ given the innovative nature of this drug and the  
90 absence of monograph available in the European pharmacopoeia. The guidelines published by the  
91 International Conference on Harmonization of Technical Requirements for Registration of  
92 Pharmaceuticals for Human Use (ICH) are not fully adapted to radiopharmaceuticals, resulting in a

93 significant disparity in previously published studies on the same subject[14–18]. The aim of this work  
94 is to describe and validate the analytical method for determining the RCP of the radiolabeled <sup>99m</sup>Tc-  
95 cAbVCAM1-5 by HPLC and TLC according to the European Association of Nuclear Medicine (EANM)  
96 recommendations. [9,13]

## 97 2. Materials and Methods

### 98 2.1. Reagents and Equipment

99 cAbVCAM1-5, the sdAb used for radiolabeling, was provided by PX'Therapeutics (France). Reagent kit  
100 for <sup>99m</sup>Tc tricarbonyl core synthesis was supplied by ERAS Labo (France). NAP 10 purification column  
101 was obtained from GE Healthcare. <sup>99m</sup>Tc eluate was provided by a <sup>99</sup>Mo / <sup>99m</sup>Tc generator (Tekcis®  
102 Curium, France).

103 Analytical HPLC was carried out using column eluting with a pump and UV detector Shimatzu LC-  
104 20AD (Kyoto, Japan) coupled with the radio detector Flow-Ram (Lablogic, Brandon, FL, USA) and  
105 controlled by the software Laura (edition 4 sp1, Lablogic, Brandon, FL, USA). Flow-Ram is a NaI  
106 detector, and the parameters for this analytical method were as follows: 850 V for photomultiplier  
107 voltage and tubing of 0.018 cm<sup>3</sup> for liquid scintillation flow cell volume.

108 The HPLC system was equipped with a Symmetry 300 C4 (Waters, 150\*4.6mm, 5µm). Concerning the  
109 UV detection, the samples were monitored with a UV detector at 200 nm and the column  
110 temperature was around 20 °C. The solvents used were (A) ultra-pure water + 0.1 % trifluoroacetic  
111 acid (TFA) and (B) acetonitrile/H<sub>2</sub>O (90:10 ; v/v) + 0.1 % TFA. TFA was purchased from Sigma Aldrich  
112 (HPLC > 99%) and acetonitrile from VWR (HPLC—super gradient Reagent Eur. Ph). The flow rate of  
113 the mobile phase was set at 1 mL/min, with a total run of 30 minutes. The following gradient was  
114 used upon HPLC analysis: 0–5 min 95% A, 5–20 min 95% A to 40% A, 20-25 min 40% A, 25-30 min  
115 40% A to 95% A. The HPLC injection volume was 20 µL.

116 The retention time (Rt) of <sup>99m</sup>Tc-cAbVCAM1-5 is 18.93 minutes, while the impurities <sup>99m</sup>TcO<sub>4</sub><sup>-</sup> and  
117 <sup>99m</sup>Tc-tricarbonyl have a retention time corresponding to the dead volume of the column, i.e. Rt=  
118 2.20 minutes.

119 Analytical TLC was carried out using the radio detector Scan-Ram (Lablogic, Brandon, FL, USA) and  
120 instant thin layer chromatography impregnated with a silicic acid (ITLC SA) strips (1 x 10 mm). Scan-  
121 Ram is a plastic scintillation detector, and the parameters for this analytical method were as follows:  
122 820 V for photomultiplier voltage and collimator of 3 mm. The mobile phase was composed of  
123 sodium chloride 0.9%. The volume of sample deposited on the strip was 5 µL.

124 <sup>99m</sup>Tc-cAbVCAM1-5 remains at the baseline, i.e, a retardation factor (Rf) between 0.0 and 0.3 and  
125 <sup>99m</sup>TcO<sub>4</sub><sup>-</sup> and <sup>99m</sup>Tc-tricarbonyl (impurities) migrates with the solvent, i.e, a Rf between 0.6 and 1.0.

### 127 2.2. Synthesis development:

128 The experimental radiopharmaceutical was produced in a specific radiopharmacy laboratory in a  
129 class A high-energy shielded enclosure. cAbVCAM1-5 labeling with <sup>99m</sup>Tc was performed as previously  
130 described [5]. Firstly, 900µL of Na<sup>99m</sup>TcO<sub>4</sub><sup>-</sup> (2.5-3 GBq) was injected into the precursor vial from the  
131 <sup>99m</sup>Tc CORE core Tricarbonyl. The vial was incubated at 100°C in a water bath (Stuart™) during 20  
132 minutes. After neutralization with HCl 1 M (200 µL), 77 µL (100 µg) of sdAb was injected in the vial  
133 and heated for 45 minutes at 75°C. After the end of the heating <sup>99m</sup>Tc-cAbVCAM1-5 was purified on a  
134 NAP 10 column and diluted to obtain a final concentration around 200 MBq/mL ± 10 %. Finally, the  
135 last stage allowed for the sterilizing filtration of the final product through 0.22 µm filters (Pall  
136 medical).

137

### 138 2.3. Validation of the analytical method for radiochemical identity

139 As there are no stable isotopes available for technetium, we used the “cold” (non-radiolabelled) sdAb  
140 cAbVCAM1-5 as a reference to assess the radiochemical identity of the experimental  
141 radiopharmaceutical. Validation of <sup>99m</sup>Tc-cAbVCAM1-5 radiochemical identity was performed by  
142 determining Rt of the radioactive product and of the “cold” reference standard (UV). Both must have  
143 identical Rt with an 5 % margin of error as the UV and radioactivity detector are generally connected  
144 in line [13]. The delay time between the non-radioactive reference standard and the radioactivity  
145 detector was determined and represented 0.1 min.

146

### 147 2.4. Validation of analytical method – Radiochemical purity

148 The validation of the analytical method for the determination of <sup>99m</sup>Tc-cAbVCAM1-5 RCP was carried  
149 out according to the EANM ~~guidelines~~ [13], the ICH Q2 guidelines [19] and the guide for the  
150 elaboration of monographs on radiopharmaceutical preparations [9].

#### 151 2.4.1. Specificity

152 Specificity confirmed the ability of the analytical method to provide an unequivocal evaluation of the  
153 active substance in the presence impurities that could be found in the final product. For this purpose,  
154 we evaluated the Rt for HPLC and the Rf for TLC of the main substance (<sup>99m</sup>Tc-cAbVCAM1-5) and of  
155 the impurities (free <sup>99m</sup>TcO<sub>4</sub><sup>-</sup> and <sup>99m</sup>Tc-tricarbonyl) three times. As the two impurities had the same  
156 retention time and retardation factor, we used the <sup>99m</sup>TcO<sub>4</sub><sup>-</sup> impurity for this ~~analyse~~ analysis. The  
157 specificity was thus evaluated by calculating the resolution:  $R_{SHPLC} = 2 \times \frac{(Rt_2 - Rt_1)}{(W_2 + W_1)}$  and  $R_{STLC} = 2 \times$   
158  $\frac{(Rf_2 - Rf_1)}{(W_2 + W_1)}$  (W represented the peak width to baseline). Rs should be higher than 2 for both methods,  
159 HPLC and TLC.

#### 160 2.4.2. Accuracy

161 Four samples of <sup>99m</sup>Tc-cAbVCAM1-5 were spiked with known amounts of <sup>99m</sup>TcO<sub>4</sub><sup>-</sup> impurity and the  
162 exact <sup>99m</sup>TcO<sub>4</sub><sup>-</sup> content was assessed. Standard radio TLC and HPLC analysis were performed on each  
163 sample. Each sample were analysed twice and the average <sup>99m</sup>TcO<sub>4</sub><sup>-</sup> was used to determine:

- 164 - Recovered RCP (= 100 – (% measured free <sup>99m</sup>TcO<sub>4</sub><sup>-</sup>).
- 165 - Theoretical RCP  $\left( = \frac{RCP\ original * (100 - (\% added\ free\ ^{99m}TcO_4^-))}{100} \right)$  (with RCP original =  
166 radiochemical purity of the original sample)
- 167 - Percentage recovery according the following formula: ( $\% recovery = \frac{(recovered\ RCP)}{(theoretical\ RCP)} \times$   
168 100)

169 The considered acceptable recovery criteria was in the 90–110 % [13].

170

#### 171 2.4.3. Repeatability and intermediate precision

172 Repeatability:

173 Six TLC plates and six HPLC runs of the same sample produced by one <sup>99m</sup>Tc-cAbVCAM1-5 synthesis  
174 were used to determine the precision of the method. For each chromatogram obtained, we

175 integrated the peaks corresponding to <sup>99m</sup>Tc-cAbVCAM1-5 and impurities to obtain the Rf or Rt and  
176 the RCP. Standard deviation (SD) and coefficient of variation (CV) values were calculated.

177 Intermediate precision:

178 A same sample of <sup>99m</sup>Tc-cAbVCAM1-5 was analysed by three different operators three times. For TLC,  
179 each operator had to deposit the sample, place the strip in the development tank, scan the plate and  
180 finally evaluate the radiochromatogram. For HPLC, each analyst had to inject the sample and analyse  
181 the radiochromatogram. For each chromatogram obtained, we integrated the peaks corresponding  
182 to <sup>99m</sup>Tc-cAbVCAM1-5 and impurities to obtain the Rf or Rt and the RCP. SD and CV values were  
183 calculated.

#### 184 2.4.4. Linearity

185 In order to assess linearity, we used the statistical function of linear regression with the least squares  
186 method. The equation of the curve and the correlation coefficient were calculated according to the  
187 equation  $y = ax + b$ , where  $y$  is the area under the peak curve of the active substance expressed in  
188 count per second (cps) ;  $a$  is the slope of the line,  $x$  is the volumetric radioactive activity of our  
189 sample expressed in MBq/mL and  $b$  the intersection of the line with the y-axis. Given the relatively  
190 short half-life of technetium-99m, we considered that the radioactive decay of the tracer was  
191 sufficient to assess linearity. To achieve this, we injected the sample of <sup>99m</sup>Tc-cAbVCAM1-5 in HPLC or  
192 deposited it on a strip for TLC method, at different time over a period of 8 hours. The correlation  
193 between the radioactive concentration and the area of the main peak was then evaluated and the  
194 correlation coefficient ( $R^2$ ) calculated.

#### 195 2.4.5. Robustness

196 The assessment of robustness measures the ability of the method to remain unaffected by small but  
197 deliberate variations in the parameters of the method and gives an indication of its reliability during  
198 normal use.

199 Two variations of methods were used to evaluate this parameter in HPLC:

- 200 - Condition A : samples were injected with a different column for HPLC (Column C18)
- 201 - Condition B : HPLC mobile phase modification :
  - 202 o (A) ultra-pure water + 0.1% trifluoroacetic acid (TFA)
  - 203 o (B) acetonitrile + 0.1 % TFA instead of acetonitrile/H2O (90:10 ; v/v) + 0.1 % TFA

204 To evaluate this parameter in TLC, we performed 2 different conditions

- 205 - Condition A : Modification of the migration strip, ITLC impregnated with a silica gel (ITLC SG)  
206 instead of ITLC SA (with silicic acid).
- 207 - Condition B : Using NaCl 20 % instead of NaCl 0.9 % as the mobile phase

208 For each method and each condition we evaluated the Rt and the Rf and the RCP of <sup>99m</sup>Tc-  
209 cAbVCAM1-5

#### 210 2.4.6. Limit of quantification

211 The quantification limit (LOQ) was determined using the signal-to-noise ratio approach, where  
212 known low concentrations of free technetium (<sup>99m</sup>TcO<sub>4</sub><sup>-</sup>) impurity were compared to the background  
213 noise. A signal-to-noise ratio of 10:1 was used to assess the quantification limit. We prepared  
214 samples of 1% to 10 % of the final activity, which was usually around 200 MBq/mL, and injected each  
215 dilution twice in the HPLC and three times in the TLC.

216 2.4.7. Range

217 The applicable measurement range was defined based on the assessment of linearity and limit of  
218 quantification. The upper activity limit is determined by the intrinsic characteristics of detector  
219 response, which is known to be linear provided that deadtime does not exceed 5 % [13].

220 3. Results and discussion:

221 3.1. Validation of the analytical method: Radiochemical identity:

222 The radiochemical identity test confirmed that the  $R_t$  of the major peak of the radioactive product  
223 ( $^{99m}\text{Tc-cAbVCAM1-5}$ ) matched with the  $R_t$  of the cold reference standard (cAbVCAM1-5) (figure 1).  
224 This test could only be performed using HPLC method. Under the HPLC analytical conditions  
225 described above, we found a difference of 0.68 minutes (corresponding to 96.41 % of the radiotracer  
226 retention time). The difference between retention times was therefore no more than 5 %. We could  
227 imagine that this retention time would be even closer if it were possible to obtain stable technetium,  
228 which was not possible here.

229

230 3.2. Validation of the analytical Method (TLC and HPLC): Radiochemical purity

231 3.2.1. Specificity

232 The specificity of the analytical method was assessed by comparing the retention times of  $^{99m}\text{Tc-}$   
233  $\text{cAbVCAM1-5}$  (radiotracer) and the potential impurities found: free  $^{99m}\text{TcO}_4^-$ . The resolution between  
234 the impurities and the  $^{99m}\text{Tc-cAbVCAM1-5}$  peak was  $R_{S_{HPLC}} = 2 \times \frac{(R_{t2}-R_{t1})}{(W_2+W_1)} = 45.01$  (Table 1). As this  
235 value was well above the reference, we can conclude that this HPLC analysis method was specific for  
236 correctly separating potential impurities from the radiotracer peak. For the TLC method, the  
237 resolution between the  $^{99m}\text{TcO}_4^-$  impurity and the  $^{99m}\text{Tc-cAbVCAM1-5}$  peak was  $R_{S_{TLC}} = 2 \times$   
238  $\frac{(R_{f2}-R_{f1})}{(W_2+W_1)} = 12.73$  (Table 1). This result meets the acceptance criteria ( $R_s > 2$ ) and therefore once again  
239 we can conclude that the thin layer chromatography system is perfectly suitable for separating  
240 impurities from the investigational radiopharmaceutical.

241

242 3.2.2. Accuracy

243 For this test, it is essential to prepare the samples carefully to ensure precision. To achieve this,  
244 accurate pipetting and radioactivity measurement must be carried out as precisely as possible.  
245 Recovery percentages were determined using the calculated and theoretical RCP for each sample.  
246 The results were conclusive for HPLC method (Table 2), with values ranging from 99.52 to 101.40 %,  
247 well within the specifications (i.e. between 90 and 110 %). For the TLC detector, the range of  
248 recovery percentage values was 99.51 % to 101.97 % (Table 2), which complied equally with the  
249 specifications. This method can be used when the main radioactive impurity is known. This is the  
250 case here with the  $^{99m}\text{TcO}_4^-$  impurity, which is usually found during technetium-99m radiolabelling. It  
251 is particularly interesting to highlight that the possibility of combining the HPLC method with the TLC  
252 method confirms the absence of adsorption of impurities in the HPLC system. Similar results were  
253 found for the two analytical methods for the spike recovery analysis, confirming the accuracy of the  
254 measurement.

255

256 3.2.3. Repeatability and intermediate precision

257 The precision of the radio detector was determined by repeatability and intermediate precision.

258 The results obtained in table 3 concerning the retention time of the <sup>99m</sup>Tc-cAbVCAM1-5 product and  
259 the RCP were consistent for repeatability and intermediate precision with coefficients of variation of  
260 less than 2 %. The HPLC analytical method therefore allows the generation of repeatable results for  
261 the assessment of Rt of compounds and PRC. The precision of the TLC method was also assessed by  
262 repeatability and intermediate fidelity. The results obtained (table 3) were consistent with the  
263 specifications for radiochemical purity, since the coefficients of variation for repeatability and  
264 intermediate precision were below 2%. With regard to the retention factor, the CVs were greater  
265 than 2% for both conditions. This may be due to a non-reproducible strip migration time or different  
266 strip sizes. However, this does not affect the determination of the RCP, as the resolution remained  
267 well above 2. Under these conditions, we have concluded that the analytical method by TLC allows us  
268 to generate precise results for evaluating the RCP. It is interesting to underline that the intermediate  
269 precision test is perfectly suited to assessing the precision of results obtained with 3 different  
270 operators, since in HPLC method the compound is manually injected into the system, and in TLC  
271 method there may be differences in sample handling (sample removal, migration of the plate in the  
272 tank, or TLC plate development on the radiochromatograph).

273 3.2.4. Linearity

274 The linearity of HPLC and TLC radiodetectors has been validated by injecting or depositing the sample  
275 several fold (decreasing with time). Knowing the initial volume concentration, we can calculate the  
276 theoretical volume concentration at different times. We then evaluated the correlation between the  
277 area under the curve of the peak of interest and the volume concentration of the sample tested.  
278 Under these conditions, the linear regression line enabled us to determine the linear correlation  
279 coefficient, which was equal to  $R^2 = 0.995$  for the HPLC method and  $R^2 = 0.998$  for the TLC method  
280 (figure 2). For both methods, the linearity of radioactivity measurement systems were well  
281 demonstrated. ~~A dilution method was also used, but the quality and reproducibility of the pipetting~~  
282 ~~was not sufficiently satisfactory. We therefore opted to measure linearity based on the technetium-~~  
283 ~~<sup>99m</sup>Tc decay.~~ A dilution method was also used, but the quality and reproducibility of pipetting lead to  
284 an additional bias. For this reason, we validated the assay by simple technetium-99m decay, as  
285 recommended by the EANM guideline[13].

286

287 3.2.5. Robustness

288 To assess the robustness of our HPLC method, we first changed chromatographic column. We used a  
289 C18 column instead of the C4 column generally selected. In this case, the retention time was reduced  
290 ( $18.48 \pm 0.01$  min). The profile of the chromatogram obtained was also modified, with a broad peak  
291 that dropped off slowly. We also observed increased retention within the column because the  
292 intensity of the peak was lower than that normally observed. For the second condition, we modified  
293 the mobile phase by increasing the proportion of organic solvent. In this case we obtained a  
294 chromatogram with a shorter retention time for the <sup>99m</sup>Tc-cABVCAM1-5 peak than usually found  
295 ( $17.88 \pm 0.01$  min), which was consistent with the increase of the acetonitrile proportion. For each of  
296 the two conditions, the ~~PRC~~ RCP assessment was not affected ( $98.81 \pm 0.11$  % for condition A and  
297  $99.68 \pm 0.08$  % for condition B) with a CV of 0.08 % and 0.11 % for conditions A and B.

298 To assess the robustness of the TLC method, we first used a glass microfiber chromatography paper  
299 impregnated with a silica gel (ITLC-SG) instead of glass microfiber chromatography paper



300 impregnated with a silicic acid (ITLC-SA) usually used. Switching to a different strip (ITLC SG instead of  
301 ITLC SA) did not affect the retardation factor of the radiotracer peak ( $0.09 \pm 0.03$ ), or the assessment  
302 of RCP ( $97.37 \pm 0.13$  %). For the second condition, modification of the mobile phase (20% NaCl  
303 instead of 0.9% NaCl) did not alter the retention factor of the peak of interest ( $0.13 \pm 0.03$ ), nor did it  
304 affect the PRC evaluation ( $99.29 \pm 0.22$  %). For each of the conditions tested and in both methods,  
305 the CV obtained for PRC assessment were less than 2%. (HPLC conditions A: CV= 0.08 % and  
306 condition B: CV= 0.11 % ; TLC condition A: CV= 0.13 % and condition B: CV= 0.23 %). These results  
307 confirm the robustness and reliability of the two analytical methods developed.

308 Even though robustness assessment is not listed in the EANM recommendation. We nevertheless  
309 decided to evaluate this parameter as recommended in the guide for the elaboration of monographs  
310 on radiopharmaceuticals preparation[9].

#### 311 3.2.6. Limit of quantification

312 The limit of quantification was measured by diluting a sample of  $^{99m}\text{TcO}_4^-$  until a signal-to-noise ratio  
313 of around 10:1 was obtained. We obtained a LOQ for the HPLC method of 2.10 MBq/mL (S/N=  
314 11.5:1) and 2.0 MBq/mL (S/N 7.1:1) for the TLC method. These values correspond to approximately 1  
315 % of the usual radioactive volumetric activity. Linear regression of the integrated peak areas  
316 obtained against the calculated radioactive concentration gave a correlation coefficient of 0.998 for  
317 the HPLC method and 0.996 for the TLC method. This also confirmed the linearity of the two  
318 radiodetectors for low and even very low radioactive concentrations. Both linear regressions are  
319 shown in figures 3.

#### 320 3.2.7. Range

321 The validated analysis range was between the value found for the LOQ (2.1 MBq/ mL for the HPLC  
322 method and 2.0 MBq/mL for the TLC method) and the high value of the linearity test (202.0 MBq/mL  
323 for HPLC method and 228 MBq/mL for TLC method). This range was selected to meet the validation  
324 guidelines by EDQM and our synthesis specification. Table 4 is a summary of the results obtained for  
325 the HPLC and the TLC methods concerning the analytical validation of the RCP of  $^{99m}\text{Tc-cAbVCAM1-5}$ .  
326 The results obtained are consistent with the specifications for all the parameters except for the  
327 assessment of the retardation factor of the main peak in TLC method for both repeatability and  
328 intermediate precision. However, this does not affect the evaluation of the RCP because the  
329 specificity for each analysis was always optimal ( $R_s > 2$ ).

### 330 4. Conclusion

331 Analytical method validation for radiopharmaceuticals is very specific and requires adaptation of the  
332 ICH Q2 recommendations. This manuscript describes the validation of an HPLC and TLC method for  
333 determining the radiochemical purity of the investigational radiopharmaceutical  $^{99m}\text{Tc-cAbVCAM1-5}$ .  
334 In accordance with EANM and European Pharmacopoeia recommendations, we validated the  
335 parameters of specificity, accuracy, linearity, precision with repeatability and intermediate precision,  
336 limit of quantification, robustness and range for each of the two methods (TLC method and HPLC  
337 method). The advantage of this study is the ability to validate the analytical methods using liquid and  
338 solid chromatography (independent and complementary methods), thereby confirming the results  
339 obtained and avoiding any problems with the adsorption of radioactive impurities on the HPLC  
340 column. This therefore allows us to correctly assess the radiochemical purity of  $^{99m}\text{Tc-cAbVCAM1-5}$ , a  
341 new radiotracer targeting inflammation in vulnerable plaques. Partly thanks to this work, the French  
342 national agency for the safety of medicines and health products was able to validate the IMPD for  
343 this experimental radiopharmaceutical, enabling a phase I-IIa clinical trial to be carried out

344 (NCT04483167). In addition, this manuscript may be of future use to teams wishing to validate their  
345 analytical method for assessing the radiochemical purity of new radiopharmaceutical drugs under  
346 development.

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#### 348 **CRedit authorship contribution statement**

349 **Juliette Mutin:** Conceptualization, Validation, Formal analysis, investigation, Validation, Writing –  
350 original draft. **Pauline Orhon:** Conceptualization, Methodology, Validation, Formal analysis,  
351 investigation, Validation, Writing – original draft. **Luc Choisnard:** Writing - Review & Editing. **Maxime**  
352 **Lassiaz:** Conceptualization, Validation, Formal analysis, investigation, Validation, Writing – Review &  
353 Editing. **Sandrine Bacot:** Methodology, Resources, Writing – Review & Editing. **Mitra Ahmadi:**  
354 Methodology, Resources, Writing – Original Draft. **Nicolas De Leiris:** Conceptualization,  
355 Methodology, Writing - Review & Editing. **Loic Djaileb:** Conceptualization, Methodology, Writing -  
356 Review & Editing. **Alexis Broisat:** Conceptualization, Resources, Writing - Review & Editing,  
357 Visualization, Supervision, Funding acquisition. **Catherine Ghezzi:** Resources, Writing - Review &  
358 Editing, Visualization, Supervision, Funding acquisition, Project administration. **Pierrick Bedouch:**  
359 Resources, Writing - Review & Editing, Visualization, Supervision. **Marie Dominique Brunet:**  
360 Conceptualization, Resources, Writing - Review & Editing, Visualization, Supervision, Project  
361 administration. **Julien Leenhardt:** Conceptualization, Methodology, Validation, Formal analysis,  
362 Investigation, Validation, Writing – original draft, Visualization.

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#### 367 **Declaration of competing interest**

368 The authors declare that they have no known competing financial interests or personal relationships  
369 that could have appeared to influence the work reported in this paper.

#### 370 **Data availability**

371 Data will be made available on request

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