Short note

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^{99m}Tc-aprotinin – optimisation and validation of radiolabelling kits for routine preparation for diagnostic imaging of amyloidosis

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Technetium-99m aprotinin was prepared from an optimised radiolabelling kit formulation containing aprotinin, alkaline buffer and stannous chloride (reducing agent) and radiolabelled using ^{99m}Tc-pertechnetate. The labelling was achieved within 25 min, with radiochemical purities of >98%.

Keywords: ^{99m}Tc-aprotinin; HPLC; Validation; amyloidosis; Aprotinin; Technetium-99m

Introduction

Aprotinin (trade name Trasylol®) is a small (6512 Da) serine protease inhibitor obtained from bovine lung. Aprotinin radiolabelled with technetium-99m is used for diagnostic imaging of patients with suspected amyloidosis, a disorder of protein metabolism resulting from extracellular deposition of the protein amyloid, using single-photon emission-computed tomography.[1–4] ^{99m}Tc-aprotinin binds to amyloid fibrils of varying chemical composition *in vivo*.[5] In 2005, Smyth and Tsopelas[6] reported the development of a radiolabelling kit for the preparation of ^{99m}Tc-aprotinin. The kit was prepared from a formulation containing Trasylol (aprotinin), alkaline buffer and stannous chloride (reducing agent). In our hands, these kits gave somewhat inconsistent results in terms of radiochemical purity of the ^{99m}Tc-aprotinin product; thus, we attempted to optimise this formulation in order to improve the reliability of the procedure.

The Cys^{14}-Cys^{38} disulphide bridge in aprotinin is readily cleaved by reducing agents,[7] and we believe that this disulphide bridge is reduced with stannous chloride in the radiolabelling kit along with reduction of ^{99m}Tc-pertechnetate, thus enabling technetium-99m to be bound covalently to one of the free cystines as Tc(V) or Tc(IV)[5]; it is well known that the thiol group shows a great tendency to bind to technetium forming stable complexes.[7] Therefore, it is important that the disulphide bridge in aprotinin is reduced to enable labelling of the protein with technetium-99m.

The aim of this study was to optimise and validate the formulation of the aprotinin radiolabelling kit in order to give reliably high radiochemical purity ^{99m}Tc-aprotinin. For technetium-99m labelling of pharmaceuticals, the reduction of ^{99m}Tc-pertechnetate to a lower oxidation state is prerequisite to achieve a high labelling efficiency. Several factors may influence this reduction, such as the amount of reductant and ligand, pH and temperature. During reduction in an aqueous solution, the ligand can stabilize the lower oxidation state to prevent the formation of ^{99m}Tc-colloid ($^{99m}TcO_2$).[5] It is important to use a balanced amount of stannous chloride. A minimum concentration of stannous ions is required to ensure that aprotinin remains in a reduced state in solution, whilst a large excess of stannous ions may cause an undesirable side reaction between tin and technetium, leading to the formation of a mixed metal complex.[5]

Results and discussion

The radiolabelling kits were initially prepared as described by Smyth and Tsopelas,[6] but the radiochemical purity of the product was found to vary from 89–97%. On development of an HPLC analysis method for quantification of aprotinin, we discovered that this method was able to distinguish partially reduced aprotinin (where the Cys^{14}-Cys^{38} disulphide bridge is cleaved) from intact aprotinin and thus was applicable for analysis of the kit formulation, with a view to optimising the relative amount of partially reduced aprotinin (Figure 1). Directly after preparation, kits were analysed by HPLC to follow the reduction of aprotinin over time. It was clear from these experiments that aprotinin was slowly reduced over a period of around 75 min (Figure 2). On increasing the amount of tin(II) chloride reductant from 180 (amount reported by Smyth and Tsopelas) to 280 μg/ml, aprotinin was reduced more rapidly (Figure 2). Using these two concentrations of reductant in the radiolabelling kits, we prepared ^{99m}Tc-aprotinin and measured the radiochemical purity by iTLC. For the two reductant concentrations, average radiochemical purities were 95.5% and 98.2%, respectively. Kowalsky *et al.*[5] reported that tin(II) loss occurs rapidly in solution (15% in 1 h) if not continuously

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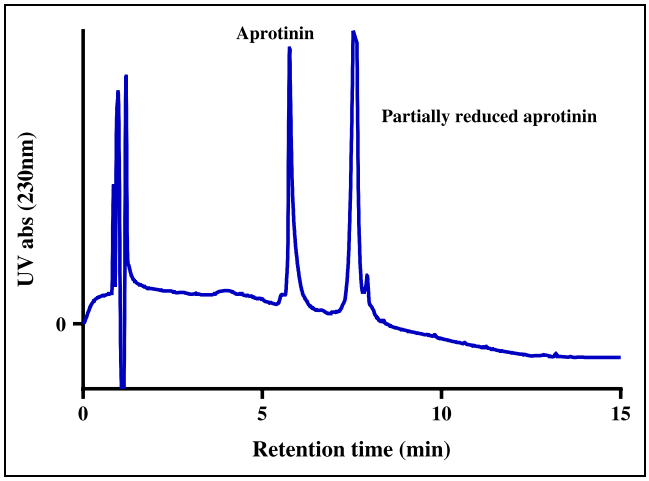

Figure 1. Ultraviolet (UV) chromatogram (UV-abs 230 nm) of an aprotinin radiolabelling kit.

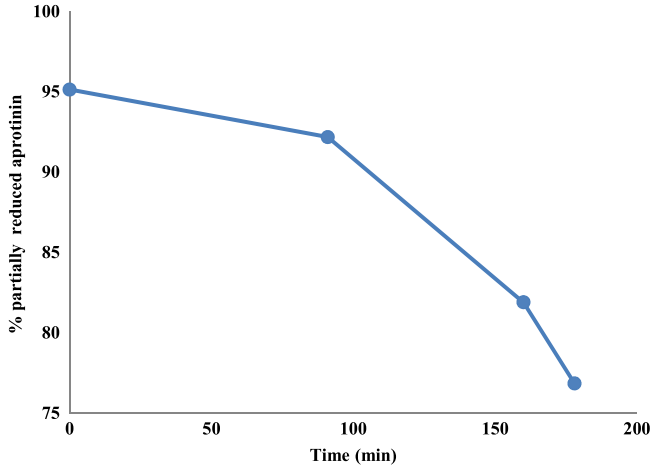

Figure 3. The figure shows that partially reduced aprotinin is readily oxidized back to its native form on exposure to oxygen (bubbling air through the solution). After 3 h, the amount of partially reduced aprotinin has fallen to 77%.

protected with a nitrogen atmosphere. Oxidation of tin(II) ions decreases the reducing power of the kit. The extra tin used will act as a buffer against small amounts of oxygen and thus simplify the preparation of the radiolabelling kits by avoiding the necessity of a fully inert atmosphere, for example, by using a nitrogen-filled glove-box. It is known that partially reduced aprotinin is readily oxidized back to aprotinin on exposure to air (oxygen).[8] Using our HPLC method, we verified this by slowly bubbling air into the radiolabelling kit formulation (with an amount of partly reduced aprotinin >98%) over a period of 3 h (Figure 3). The increased tin concentration in the radiolabelling kits will help to protect against this process and thus improve the radiolabelling efficiency. However, further radiolabelling experiments using radiolabelling kit formulations with varying amounts of partially reduced aprotinin demonstrated that a high degree of partial reduction is not critical for obtaining ^{99m}Tc-aprotinin in high radiochemical purity and that the concentration of reductant is most probably more critical for reducing ^{99m}Tc-pertechnetate to an oxidation state amenable to radiolabelling. Surprisingly, we found that only around 14% of partially reduced aprotinin was necessary to ensure the preparation of a product of high radiochemical purity (>90% to comply with the quality control requirements, Figure 4). It should be noted that in this experiment, the extent of partial reduction was measured prior to radiolabelling for each sample and, due to this delay (8–12 min) along with the time allowed for radiolabelling, the amount of partial reduction of aprotinin was most probably higher in the radiolabelling solution than indicated in Figure 4. The amount of partially reduced aprotinin present can be used as an indication of the presence of oxygen in the kit formulation. Further increases in tin concentration did not appear to further improve the radiochemical purity of ^{99m}Tc-aprotinin, so the kit formulation with 280 μg/ml tin(II) chloride is considered to be optimal.

We attempted to use our new HPLC method to measure the radiochemical purity of ^{99m}Tc-aprotinin, but the majority of the radioactivity adhered to the injection system and the column, thus making this method unsuitable. Chromatographic recovery was determined separately for ^{99m}Tc-aprotinin, ^{99m}Tc-pertechnetate, ^{99m}Tc-PYP and ^{99m}Tc-colloid, by comparing the amount of radioactivity injected on to the system and the amount of radioactivity in the column eluate. The recoveries for ^{99m}Tc-aprotinin, ^{99m}Tc-pertechnetate, ^{99m}Tc-PYP and ^{99m}Tc-colloid were 32%, 83%, 41% and 23%, respectively. Millar *et al.*[9] described similar challenges in the development of an HPLC method for analysis of RCP of ^{99m}Tc-succimer injection, where technetium-99m impurities were adsorbed in the stainless-steel sample loop of the HPLC system.

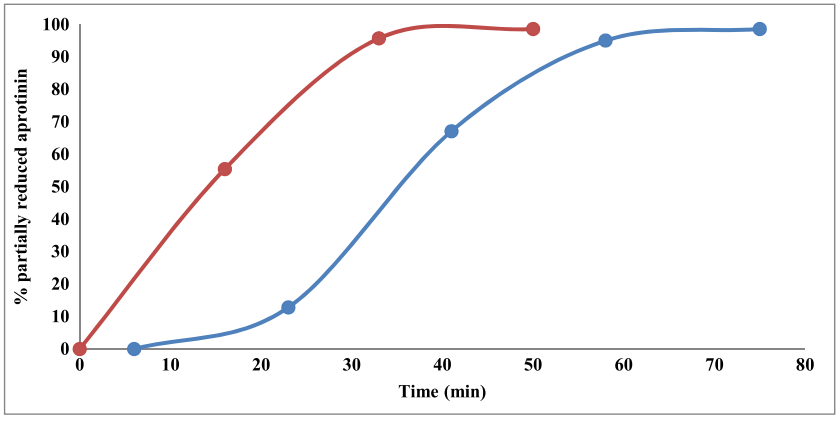

Figure 2. Formation of partly reduced aprotinin over time using 180 (blue) and 280 μg/ml (red) stannous chloride as reducing agent.

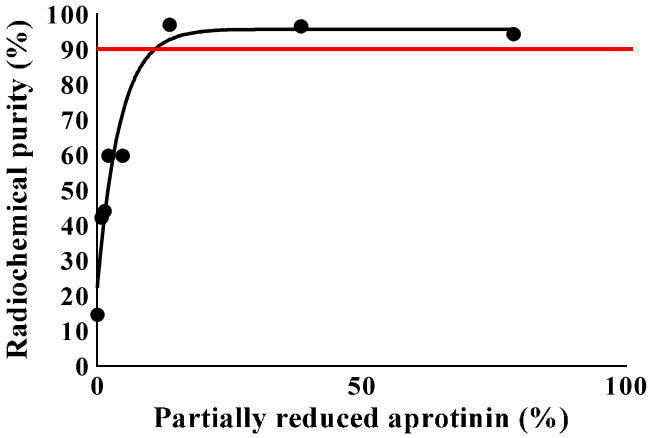

Figure 4. The relationship between percentage partly reduced aprotinin in the radiolabelling kit and the radiochemical purity of ^{99m}Tc-aprotinin is shown. The minimum requirement for the radiochemical purity of ^{99m}Tc-aprotinin of 90% is shown (red line). It should be noted that there was a delay between the measurement of partially reduced aprotinin with HPLC and the radiolabelling procedure and therefore the amount of partly reduced aprotinin was most probably higher in the radiolabelling solution than indicated here.

Table 1. Acceptance criteria for ^{99m}Tc-aprotinin

^{99m}Tc-aprotinin acceptance criteria	
pH	8.5–9.7
^{99m}Tc-pertechnetate	<5%
^{99m}Tc-PYP	<5%
$^{99m}TcO_2$	<10%
Radiochemical purity	≥90%
Bacterial endotoxins	<1.0 EU/ml
Test for sterility	Complies

Quality control

For routine preparation of radiolabelling kits, we verified the content of aprotinin and the extent of reduction by HPLC, and the tin concentration using a colourimetric method. All batches were tested for bioburden prior to sterile filtration and complied with the recommended limits of <10 cfu/100 ml.

For quality control of the formulated product, ^{99m}Tc-aprotinin, we used three TLC methods, as previously described.[5] The major radiochemical impurity was ^{99m}Tc-colloid (1.5–1.7%), whilst amounts of $^{99m}TcO_4^-$ and ^{99m}Tc-PYP were generally <0.5%. The acceptance criteria for ^{99m}Tc-aprotinin are shown in Table 1.

Stability

For assessment of the stability of radiolabelling kits, vials were removed at intervals of 1, 2, 3 and 6 months and tested as described previously for quality control of radiolabelling kits. A test radiolabelling was also performed, and the resultant ^{99m}Tc-aprotinin product was tested for radiochemical purity, pH, sterility and bacterial endotoxin content.

Stability results confirmed that radiolabelling kits stored at −80 °C have a shelf-life of at least 6 months and can be used for preparation of ^{99m}Tc-aprotinin that meets all the acceptance criteria. Furthermore, it was demonstrated that solutions of ^{99m}Tc-aprotinin can be stored for 4 h prior to use with minimal loss of radiochemical purity (Table 2).

Table 2. Process validation batch results including 4-h stability

^{99m}Tc-aprotinin	0 h (n = 15)	4 h (n = 14)
pH	9.24 ± 0.2	9.18 ± 0.2
^{99m}Tc-pertechnetate (%)	0	0
^{99m}Tc-PYP (%)	0.01 ± 0.04	0.02 ± 0.08
$^{99m}TcO_2$ (%)	2.30 ± 1.8	4.73 ± 2.4
Radiochemical purity (%)	97.6 ± 0.98	95.3 ± 2.4

Experimental

Materials

TechneScan™ PYP™ was purchased from Mallinckrodt Medical, Holland; glycine was purchased from Sigma Aldrich, Denmark; sodium dihydrogen phosphate was purchased from Fagron A/S Denmark and Trasylol was purchased from Bayer A/S, Denmark.

All solutions used for the preparation of radiolabelling kits were prepared as described by Smyth and Tsopelas,[6] except the acidic Sn-PYP. Sn-PYP acidic solution was prepared by dissolving and combining the contents of three TechneScan™ PYP™ vials (in 3.4 ml of 0.05 M HCl), giving a stannous chloride concentration of 2.8 mg/ml.

Analysis

HPLC analysis was performed on a Thermo Scientific Ultimate 3000 HPLC system with inline Photodiode Array Detector (PDA) and radioactivity detectors. Chromatographic separation was performed using an Aeris PEPTIDE® XB-C18 column (3.6 μm, 100 Å, 150 × 4.6 mm, Phenomenex) and gradient elution with aqueous 0.1% trifluoroacetic acid (TFA) (A) and acetonitrile (B) as mobile phases (0–2 min 8% B, 2–11 min 8–60% B, 11–14 min 60% B, 14–16 min 60–8% B); flow rate 1.5 ml/min; ultraviolet detection at 230 nm and injection volume: 30–100 μl. A certified aprotinin reference standard was used (Sigma-Aldrich). For tin analysis, a commercially available colourimetric test kit was used[10] (measurement range: 0.10–2.50 mg/l), and measurements were performed using a Spectroquant® spectrophotometer (Merck Millipore). TLC strips (ITLC-SG chromatography paper, Agilent Technologies) were measured using a ScanRam radio TLC scanner (LabLogic, UK) and an Instant Imager (Packard, A Canberra Company, USA).

Radiolabelling kit preparation

Radiolabelling kits were prepared in a laminar air flow bench (class A). To a nitrogen-filled sterile, 50 ml vial with a silicone rubber septum was added in order: 10 ml of Trasylol, 15.4 ml of glycine buffer, 3.4 ml acidic TechneScan™PYP™ solution (2.8 mg/ml stannous chloride in radiolabelling kit) and 6.2 ml sterile water. The resultant solution was degassed with a continuous flow of nitrogen gas (~100–200 ml/min) for 1 h. Following removal of a 10 ml sample for bioburden testing, the remaining solution was dispensed in 1 ml aliquots through a Millex-GV 0.22 μm sterile filter into nitrogen-filled 10 ml sterile vials with chlorobutyl rubber stoppers under a constant nitrogen flow. Some vials were removed for testing, and the remainder was stored at −80 °C.

^{99m}Tc-labelling procedure

^{99m}Tc-pertechnetate was eluted from an Ultra-Technekow FM 99Mo/99mTc generator (Mallinckrodt Medical, The Netherlands). A frozen radiolabelling kit was thawed and diluted with 2 ml saline. A total of 1 ml ^{99m}Tc-pertechnetate (500–900 MBq) was added to the kit vial under aseptic conditions, and the resultant solution was mixed by shaking and left at room temperature for minimum of 25 min.

Quality control of radiolabelling kits

Three separate batches of kit formulation were prepared for validation purposes. A bioburden test was performed on a 10 ml sample of each batch. For each batch, HPLC analysis was performed to verify the amount of aprotinin and determine the extent of partial reduction. Furthermore, tin content and pH were measured. A ^{99m}Tc-radiolabelling test including quality control was performed on each batch (see succeeding texts).

Quality control of ^{99m}Tc-aprotinin

The radiochemical purity of the ^{99m}Tc-aprotinin was determined using iTLC as previously described (Smyth and Tsopelas).[6] pH was measured using a calibrated pH meter, and samples were tested for sterility and bacterial endotoxins using European Pharmacopoeia compliant methods.

Conclusions

In conclusion, we have prepared an improved radiolabelling kit for routine preparation of ^{99m}Tc-aprotinin with high radiochemical purity. Both the kits and the radiopharmaceutical products are manufactured in compliance Good Manufacturing Practice (GMP) and have been validated. Furthermore, we have developed a novel HPLC method that can separate partially reduced aprotinin from the native protein. This method allowed us to gain a better understanding of the factors important for achieving a high radiochemical purity product and is used for routine analysis of each batch of radiolabelling kits. Surprisingly, it appears that a high percentage of partially reduced aprotinin is not critical to yield ^{99m}Tc-aprotinin in high radiochemical purity. Radiolabelling kits can be stored for up to 6 months, and this allows for simple radiolabelling of aprotinin with consistently high radiochemical purity (>98%) for diagnostic imaging of amyloidosis.

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