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Usefulness of fast protein liquid chromatography as an alternative to high performance liquid chromatography of ^{99m}Tc-labelled human serum albumin preparations[1]

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Abstract

Accurate analysis of the radiochemical purity of ^{99m}Tc-labelled preparations is usually performed using an appropriate high performance liquid chromatography (HPLC) system. For the analysis of ^{99m}Tc–HSA, the European Pharmacopoeia (Ph. Eur.) prescribes an HPLC system using silica gel for size-exclusion chromatography as the stationary phase. Analysis of ^{99m}Tc–HSA preparations with this method allows one to distinguish radiolabelled polymeric, oligomeric, dimeric and monomeric forms of albumin as well as small molecular mass impurities such as pertechnetate ($^{99m}TcO_4^-$). Due to the relatively short lifetime of this type of column, two fast protein liquid chromatography (FPLC)-type columns were evaluated for their possible usefulness for quality control of ^{99m}Tc–HSA preparations, namely Superdex 200 HR 10/30 and Superdex 75 HR 10/30 (Pharmacia, Sweden). Both columns contain a matrix produced by the covalent bonding of dextran to highly cross-linked porous agarose beads. The fractionation range of the Superdex 75 HR 10/30 column was too low to obtain an adequate separation between albumin and its polymerized forms and this column was therefore considered unsuitable for the analysis of ^{99m}Tc–HSA preparations. The Superdex 200 column afforded the same pattern and relative amounts of albumin and its polymerized forms as the silica column although the resolution obtained was somewhat lower. A major difference was the fact that $^{99m}TcO_4^-$ could not be eluted from the Superdex column but was eluted from the silica column, whereas other small molecular mass, negatively charged ^{99m}Tc complexes such as ^{99m}Tc-diethylene triamine pentaacetic acid showed an identical elution pattern on both columns. The presence of pertechnetate in ^{99m}Tc–HSA preparations, however, can easily be checked using paper chromatography (PC) and acetone as the eluent. Since the lifetime of the Superdex column is twice that of the silica column, FPLC in combination with PC can be recommended as a money-saving and efficient analytical technique for the quality control of ^{99m}Tc–HSA preparations.

Keywords: FPLC; HPLC; ^{99m}Tc–HSA; ^{99m}Tc–DMP–HSA

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1. Introduction

Technetium-99m-labelled human serum albumin (^{99m}Tc–HSA is a radiopharmaceutical commonly used for the radioisotopic evaluation of the heart function. It is generally prepared by addition of pertechnetate ($^{99m}TcO_4^-$) in saline to a commercially available lyophilized labelling kit, resulting in the reduction of the heptavalent ^{99m}Tc, followed by binding of reduced ^{99m}Tc by the protein. The nature of the bond between ^{99m}Tc and HSA is not well defined and as HSA has only poor metal binding capacity, the ^{99m}Tc–HSA complex is not very stable in vivo. Recently, a more stable ^{99m}Tc–HSA derivative has been developed by derivatization of albumin prior to labelling with a limited number of dimercaptopropionyl side chains (DMP–HSA), and this derivative binds ^{99m}Tc more strongly [1].

Possible radiochemical impurities in such ^{99m}Tc–HSA preparations include $^{99m}TcO_4^-$, due to imcomplete reduction of pertechnetate during labelling. Colloidal ^{99m}Tc($^{99m}TcO_2$) can also be formed when reduced ^{99m}Tc is not completely complexed by albumin. Diethylene triamine pentaacetic acid (DTPA) is added as stabilizer in the preparation of ^{99m}Tc–DMP–HSA and, as a consequence, ^{99m}Tc–DTPA can constitute a radiochemical impurity in this radiopharmaceutical. Finally, polymerized or aggregated forms of albumin can be formed during lyophilization or derivatization of albumin. It is important to be able to detect and quantify polymeric forms of albumin since these impurities are rapidly cleared from the circulation due to phagocytosis by the reticuloendothelial system [2], which resluts in low-quality images of the blood pool.

The originally proposed techniques for quality control of ^{99m}Tc–HSA preparations (thin-layer chromatography, paper electrophoresis, trichloroacetic acid precipitation [3–5]) only allow determination of the amount of free pertechnetate. The introduction of aqueous size-exclusion columns for high performance chromatography has significantly improved the rapid analysis and separation of proteins [6]. This high-resolution technique is capable of differentiating between polymeric, oligomeric, dimeric and monomeric forms of albumin. The European Pharmacopoeia proposes in its monograph *Technetii [^{99m}Tc] Humani Albumini Solutio Iniectabilis* to carry out the chromatographic procedure using a stainless-steel column packed with silica gel for size-exclusion chromatography. This type of column demonstrates adequate separation of ^{99m}Tc–HSA and its potential impurities but often suffers from high back-pressures resulting in a short lifetime of the column. Since the fast protein liquid chromatography (FPLC) technique was introduced in 1982, it has established itself as the preferred method for protein separation. The back-pressure of this type of column is substantially lower and the lifetime of the column is about twice that of conventional high performance liquid chromatography (HPLC) columns. Therefore, it seemed very useful to investigate whether columns designed for FPLC systems are also suitable for the analysis of ^{99m}Tc–HSA preparations. The columns used in this study contain a matrix produced by the covalent bonding of dextran to highly cross-linked porous agarose beads.

2. Experimental

2.1. Radiopharmaceuticals

^{99m}Tc–HSA was prepared by reconstitution of commercially available labelling kits (Technescan HSA, Mallinckrodt Medical, The Netherlands) with a sodium ^{99m}Tc–pertechnetate solution (eluate from a commercial generator, Ultratechnekow FM, Mallinckrodt Medical, The Netherlands) according to the instructions of the manufacturer. Derivatization of albumin with a limited number of dimercaptopropionyl side chains, purification, and subsequent labelling with ^{99m}Tc has been described elsewhere [1]. Recently, a kit formulation for the preparation of ^{99m}Tc–DMP–HSA has been developed [7]. The labelling kit contains 6.2 mg DMP–HSA, 10 mg sucrose and 5 μg $SnCl_2 \cdot 2H_2O$. ^{99m}Tc–DMP–HSA is prepared by addition of ^{99m}Tc–pertechnetate (1.85–5.55 GBq ^{99}Tc) in 1–4 ml saline to such a labelling kit, followed by 0.25 ml of a solution containing 12.5 μg $SnCl_2 \cdot 2H_2O$ and 250 μg $CaNa_3DTPA$.

2.2. Thin-layer chromatography (TLC)

5 μl of the respective ^{99m}Tc–HSA or ^{99m}Tc–DMP–HSA preparations was applied on a chromatographic strip (13 cm × 1 cm) of Whatman 4 Chr paper (Whatman International Ltd., UK) at the application point, 2 cm from the bottom. The strips were eluted with acetone and, after drying, the strips were cut at 2 cm above the application point and the activity of each part was measured using a 2 in. NaI(Tl) scintillation detector, connected to a single-channel analyser and scaler. Results were corrected for background radiation. In this system, ^{99m}Tc–HSA and ^{99m}Tc–DMP–HSA remain at the application point whereas ^{99m}Tc–pertechnetate migrates with the solvent front. The percentage of $^{99m}TcO_4^-$ in the preparation was calculated from the formula:

$$\%^{99m}TcO_4^- = \frac{\text{net counts on the upper part}}{\text{net counts on the total strip}} \times 100$$

2.3. High pressure liquid chromatography (HPLC)

The respective preparations were analysed simultaneously by both HPLC and FPLC. The HPLC system consisted of a Merck Hitachi L6200 ternary gradient pump, a Valco N6 injector (Alltech, Belgium) and a 300 mm × 7.8 mm Bio-Sil SEC 250 column (fractionation range: 1×10^4–3×10^5 Da, Bio-rad, USA).

For the FPLC system, an isocratic Gilson 303 pump (Analis, Belgium), a Valco N6 injector (Alltech, Belgium) and a 300 mm × 10 mm Superdex 200 HR 10/30 column (fractionation range: 1×10^4–6×10^5 Da, Pharmacia, Sweden) were used. In a preliminary experiment, the FPLC system was used with a Superdex 75 HR 10/30 column (fractionation range: 3×10^3–7×10^4 Da, Pharmacia, Sweden). A phosphate buffer (pH 7.0; 0.05 M) containing 0.15 M NaCl and 0.05% NaN_3 was used as the mobile phase in both systems at a flow rate of 0.6 ml min^{-1} for the HPLC system and 0.5 ml min^{-1} for the FPLC system.

Radioactivity in the effluent was monitored with a 2 in. NaI(Tl) scintillation detector connected to a single-channel analyser and integrated with a RACHEL analysis program (version 1.40, Lablogic, UK) installed on a personal computer.

3. Results and discussion

Typical chromatograms of a ^{99m}Tc–HSA prparation simultaneously obtained with the Bio-Sil SEC 250 and the Superdex 200 HR 10/30 columns are shown in Fig. 1. The same elution pattern and relative amounts of albumin and its polymerized forms were obtained on both columns although the Superdex column showed broader peak shapes which is probably due to the larger particle size of the Superdex column (13 μm vs. 5 μm for the Bio-Sil column). The fractionation range of the Superdex 75 HR 10/30 column was too low to obtain adequate separation between albumin and

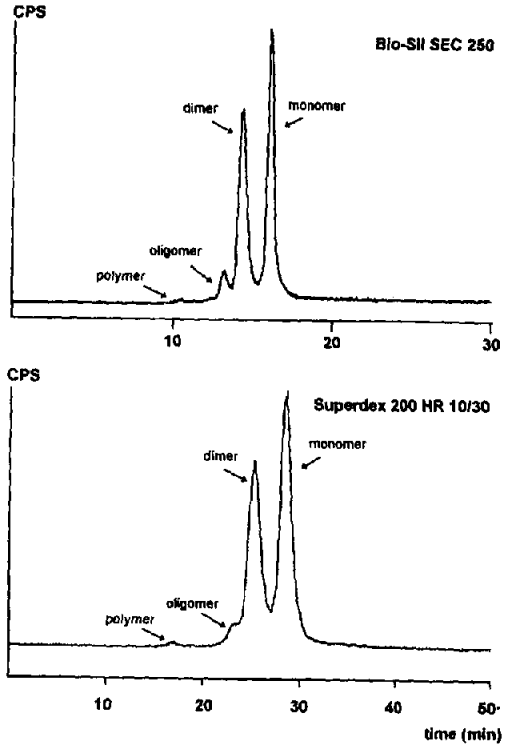

Fig. 1. Typical chromatograms of a ^{99m}Tc–HSA preparation simultaneously obtained with an HPLC-type column (Bio-Sil SEC 250) and an FPLC-type column (Superdex 200 HR 10/30).

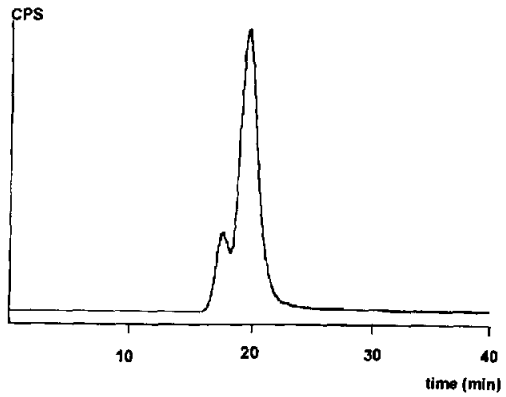

Fig. 2. Typical chromatogram of a ^{99m}Tc–HSA preparation obtained with a Superdex 75 HR 10/30 column.

its polymerized forms (Fig. 2). Therefore, this column was considered unsuitable for the analysis of ^{99m}Tc–HSA preparations and was not used further. The elution pattern of small molecules like ^{99m}Tc–DTPA was also the same on both the HPLC and FPLC (Superdex 200) columns. The main difference between the columns is the fact that $^{99m}TcO_4^-$ could not be eluted from the FPLC column whereas it was eluted from the HPLC column with a retention time (R_t) of 25 min under the described conditions. Ideally, size-exclusion chromatography should only be based on the molecular size of the analyte and should be independent of any physicochemical interaction with the stationary phase. However, it is known that non-ideal effects occur in size-exclusion chromatography [8] and the present results demonstrate a mixed-mode separation mechanism, rather than merely a molecular sieving mechanism, in which hydrophilic or electrostatic interactions cannot be neglected. Hydrophobic interactions can become important at high salt concentrations (> 1 M NaCl) [9]. Nevertheless, even when electrostatic interactions are taken into account, it remains difficult to explain why a small negatively charged molecule like $^{99m}TcO_4^-$ is reduced on the column, forming colloidal ^{99m}Tc which is not eluted from the column. However, we did not find any evidence for the presence of a reducing substance on the column.

The retention of $^{99m}TcO_4^-$ on the column does not necessarily exclude the Superdex 200 HR 10/30 column from use for analysis of ^{99m}Tc–HSA preparations since the presence of this impurity can easily and rapidly (about 5 min) be checked using paper chromatography and acetone as the eluent.

Some quantitative data on the performance of both columns are shown in Table 1. Obviously, more than twice the amount of runs could be performed using the FPLC column as compared to the HPLC column. In addition, much less variability in the retention time of the monomeric albumin peak over the lifetime of the column was observed with the FPLC column. Quantitative analysis of ^{99m}Tc–HSA preparations yielded the same results using either column, although resolution obtained with the FPLC column was somewhat lower. The resolution of both columns degraded slightly in time and this happened more quickly with the HPLC column, resulting in a markedly shorter lifetime.

4. Conclusions

The elution pattern and relative amounts of albumin and its polymerized forms are identical when a HPLC-type column (Bio-Sil SEC 250) is used for analysis of ^{99m}Tc–HSA preparations or an FPLC type column (Superdex 200 HR 10/30). The performance of both columns is not com-

Table 1
Quantitative data on the lifetime of the HPLC and FPLC columns used in this study

Parameter	Bio-Sil SEC 250 (HPLC)	Superdex 10/30 HR 200 (FPLC)
Number of runs	150	375
Mean retention time of monomeric species of albumin peak (min)	15.39 ($n = 16$)	29.40 ($n = 80$)
Day-to-day RSD (%)	9.51	2.54

pletely identical, since pertechnetate, a possible impurity in ^{99m}Tc–HSA preparations, is eluted from the HPLC column but completely retained on the FPLC column. However, paper chromatography using acetone as the eluent allows easy and rapid quantification of pertechnetate in ^{99m}Tc–HSA preparations. Since the lifetime of the FPLC column is about twice that of the HPLC column, it can be recommended to use FPLC for quality control of ^{99m}Tc–HSA preparations on condition that it is combined with paper chromatography for quantification of pertechnetate.

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