High Performance Liquid Chromatography of ^{99m}Tc Labeled Human Serum Albumin Using an *N*-Methylpyridinium Polymer Column

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Human serum albumin (HSA) labeled with ^{99m}Tc (^{99m}Tc-HSA) was analyzed by high performance liquid chromatography using a 4-vinylpyridinium polymer column which specifically resolved albumin components such as human mercaptalbumin (HMA), human non-mercaptalbumins (HNA), *etc.* The ^{99m}Tc-HSA radiochromatogram revealed that ^{99m}Tc-HSA consisted of several components. The radiochromatographic profile was similar to that of ^{99m}Tc-HMA prepared with ^{99m}Tc and separated HMA. This suggested that HMA participated mainly in ^{99m}Tc-labeling of HSA. When HSA was labeled with a stoichiometric concentration of ^{99}Tc, the HMA peak was significantly decreased and new peaks were revealed by absorbance at 280 nm. From these results, the role of HMA in labeling HSA with ^{99m}Tc was elucidated.

Keywords 99m-technetium labeled human serum albumin; mercaptalbumin; human serum albumin; technetium; pyridinium polymer; HPLC

Serum albumins are heterogeneous with respect to sulfhydryl content such as mercaptalbumin and non-mercaptalbumin.[1-3] We previously reported that *N*-methylpyridinum polymer (4VP) developed as a column packing for ion-exchange high performance liquid chromatography (HPLC) could resolve serum albumin components.[4] The characteristics of this column were effectively applied to the investigation of reactivity of *cis*-diaminedichloroplatinum (II) (an antineoplastic agent) with albumin components and the high reactivity of the sulfhydryl group in mercaptalbumin was chromatographically elucidated.[5] This suggests that the applicability of this column can be extended to the investigation of other metal–albumin complexes.

Technetium-99m (^{99m}Tc) labeled human serum albumin (^{99m}Tc-HSA) is an important radiopharmaceutical for cardiac and placenta studies. A variety of analytical methods for the ^{99m}Tc-HSA have been reported,[6-10] however, little information related to the composition or binding modes of ^{99m}Tc-HSA is available. Since technetium (Tc) has high affinity for sulfhydryl compounds,[11-13] mercaptalbumin may play a significant role in binding to ^{99m}Tc. Steigman *et al.*[14] stated that the sulfhydryl content in HSA affected the labeling efficiency of HSA with ^{99m}Tc. This study describes the application of the 4VP column to the HPLC analysis of ^{99m}Tc-HSA.

Experimental

Materials HSA was supplied by the Chemo-Serotherapeutic Research Institute (Kumamoto, Japan). Human mercaptalbumin (HMA), human non-mercaptalbumin conjugated with cysteine (HNA) and a disulfide dimer of HSA (HDA) were prepared by the method of Sogami, *et al.*[2] The sulfhydryl content of these albumins was determined by spectrophotometry with 2,2′-dithiopyridine.[15] ^{99m}Tc pertechnetate in saline solution ($Na^{99m}TcO_4$) was eluted daily from a Daiichi Radioisotope Lab. generator and 37 MBq ml^{-1} was used for each labeling reaction. Ammonium pertechnetate, $NH_4{}^{99}TcO_4$, was purchased from Amersham International plc (Buckinghamshire, U.K.). Other chemicals used were of the highest grade available.

Chromatography The HPLC system consisted of an L-6200 intelligent pump (Hitachi, Ltd., Tokyo, Japan), a low pressure gradient programmer and a Hitachi L-4000 UV detector. The column packing material, an *N*-methylpyridinium polymer cross-linked with ethylene glycol dimethacrylate, was prepared as described previously,[16] and packed into a 250 × 4 mm (i.d.) column. The labeled compound was eluted using a binary gradient from the initial buffer (solution A) of 0.05 M tris (hydroxy methyl) aminomethane (Tris)–HCl buffer (pH 7.0) to the final buffer (solution B) consisting of solution A containing 0.5 M sodium chloride at a flow rate of 0.5 ml/min (0—0.1 min, solution A from 100 to 95%; 0.1—20 min, solution A 95 to 75%; 20—25 min, solution A 75 to 0%; 25—40 min, solution A 0%). The chromatography was performed at room temperature. The eluate was monitored by a ultraviolet (UV) detector (280 nm) and a sodium iodide scintillation detector.

^{99m}Tc Labeling of Albumin ^{99m}Tc labeling of albumin was performed by the method of Nakayama *et al.*[17] in which Duolite ES-467 (Diamond Shamrock, U.S.A.) retained Sn(II) (1.8 mmol Sn/g-resin, R-Sn) was used as the reductant of $^{99m}TcO_4^-$. Albumin (3.5 mg) was dissolved in 0.5 ml of 0.01 N HCl–0.1 M NaCl and 2.5 mg of R-Sn was added. After 5 min, 0.4 ml (14.8 MBq) of $^{99m}TcO_4^-$ in saline was added and the mixture was shaken at room temperature for 30 min. The pH of the mixture was then adjusted to 7.0 with 0.066 M phosphate buffer and the final solution was passed through a 0.45 μm millipore filter. Twenty microliter samples were injected into the HPLC column.

Preparation of (^{99}Tc)-Albumin ^{99}Tc-Albumin was prepared in a manner similar to that of ^{99m}Tc-albumin. The labeling was performed using $^{99}TcO_4^-$ containing a trace amount of $^{99m}TcO_4^-$. The equivalent molar ratio of $^{99}TcO_4^-$ to albumin (3.5 mg) and 7.5 mg of reductant (R-Sn) were used.

Thin Layer Chromatography (TLC) of ^{99m}Tc-Albumin The labeled compound was developed on a thin layer cellulose strip (Merck, Germany) using a methanol–water mixture (85 : 15, v/v), then the radioactivity was measured using an Aloka chromatoscanner. Labeling efficiency was obtained using the proportion of radioactivity of albumin band to the total radioactivity on the plate.[6,18]

Polyacrylamide Gel Electrophoresis (PAGE) ^{99m}Tc-Albumin was electrophoresed on 10% polyacrylamide slab gels as described by Laemmli,[19] but without 2-mercaptoethanol and sodium dodecyl benzene sulphonate. After electrophoresis, gels were soaked in 20% ethanol, dried and autoradiographed on X-ray film (Fuji film, Tokyo Japan). Alternatively, the gel was stained with Coomassie brilliant blue R250 (CBB, Fluka, Switzerland).

Results and Discussion

In the labeling of HSA with Tc, reduction of pertechnetate ($^{99m}TcO_4^-$) is necessary, and stannous chloride has generally been used as the reductant. Since HSA has several binding sites, including sulfhydryl group for metal ions,[20-24] stannous ions (Sn(II)) in the labeling solution bind to HSA and may interfere with formation of the ^{99m}Tc-HSA complex. We previously reported that use of a macromolecular Sn(II) complex (R-Sn) as the solid phase reductant simplified the reducing process.[17] In this study, R-Sn was used as the reductant in order to minimize contamination of resulting preparations by Sn(II).

As shown in Fig. 1A, HSA is comprised of HMA, HNA and some polymeric albumins (mainly dimeric HSA). A radiochromatogram of ^{99m}Tc-HSA prepared from this HSA is also shown in Fig. 1B. The 280 nm profile of ^{99m}Tc-HSA (UV chromatogram) does not differ from that shown in Fig. 1A due to the use of the tracer scale of ^{99m}Tc. There are five major peaks at 25, 26, 28, 29 and 32 min in the ^{99m}Tc-HSA radiochromatogram. The retention times of four peaks (25, 26, 28 and 29 min) differed from those of albumin in the UV chromatogram and only the last peak at 32 min coincided with that of polymerized albumins. Under the HPLC conditions, $^{99m}TcO_4^-$ was tightly retained on the column due to anion exchange and was not eluted. Reduced ^{99m}Tc, however, was eluted at 3 min. Therefore, the radiochromatographic peaks shown in Fig. 1B would originate from ^{99m}Tc-HSA. The disagreement in the retention times of ^{99m}Tc-HSA and HSA can be explained mainly in terms of ionic charge. This also shows that ^{99m}Tc-HSA is not a single compound and is comprised of constituents with various ionic properties.

To elucidate the effect of each component on the labeling of ^{99m}Tc-HSA, HMA, HNA and HDA were prepared separately and labeled with ^{99m}Tc. Steigman *et al.*[14] stated that the sulfhydryl levels of HSA affected the labeling efficiency with ^{99m}Tc. The sulfhydryl contents and labeling efficiency of albumins are summarized in Table I. The labeling efficiency of HNA was the lowest among the albumins tested. Despite the low sulfhydryl content, however, HDA was labeled in high efficiency. These ^{99m}Tc-labeled albumins were also examined by PAGE, and the radioactive bands coincided with the band stained with CBB.

As shown in Fig. 2, ^{99m}Tc-HMA showed a similar radiochromatographic profile to that of ^{99m}Tc-HSA. However, there were indistinct peaks in the ^{99m}Tc-HNA radiochromatogram (Fig. 3). ^{99m}Tc-HDA eluted at the identical retention time (32 min) of HDA in the UV chromatogram (Fig. 4). From the radiochromatographic results, it was thought that HMA played an important role in the ^{99m}Tc labeling of HSA, and that the peak which eluted at 32 min on the ^{99m}Tc-HSA and ^{99m}Tc-HMA radiochromatograms corresponded to that of ^{99m}Tc-HDA. There was also a significant level of radioactivity in dimer fraction on the ^{99m}Tc-HSA and ^{99m}Tc-HMA autoradiograms of PAGE.

The high reactivity of HMA for technetium is probably

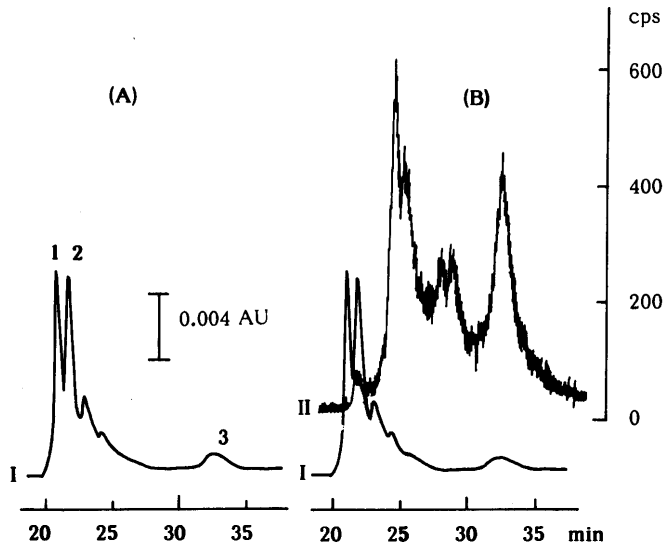

Fig. 1. Chromatograms of HSA (A) and ^{99m}Tc-HSA (B)

Peaks: 1, HMA; 2, HNA; 3, polymerized albumin. Detection; I, absorbance (280 nm); II, radioactivity. Other chromatographic conditions are in the text.

TABLE I. Sulfhydryl Content of the Albumins Used and Their Labeling Efficiency with ^{99m}Tc

Albumin	Sulfhydryl content (mol/mol albumin)	Labeling efficiency[a] (%)
HSA	0.480	95
HMA	0.935	97
HNA	0.044	77
HDA	0.086	96

a) Determined by TLC method.

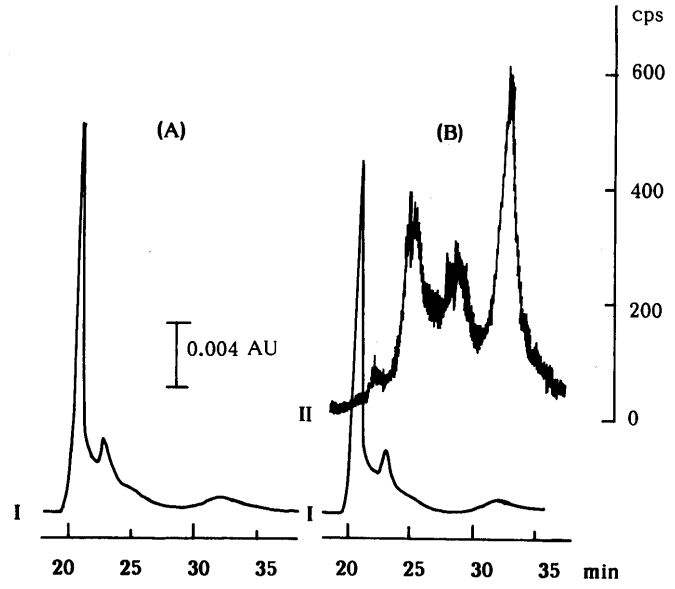

Fig. 2. Chromatograms of HMA (A) and ^{99m}Tc-HMA (B)

Chromatographic conditions are the same as those in Fig. 1.

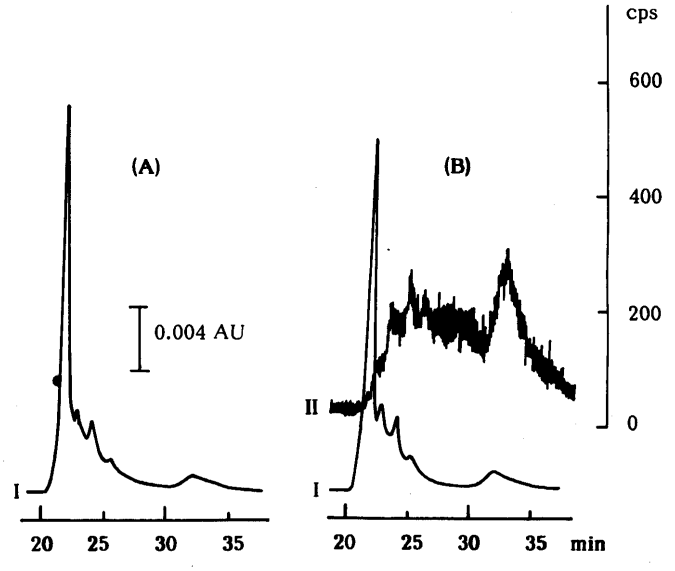

Fig. 3. Chromatograms of HNA (A) and ^{99m}Tc-HNA (B)

Chromatographic conditions are the same as those in Fig. 1.

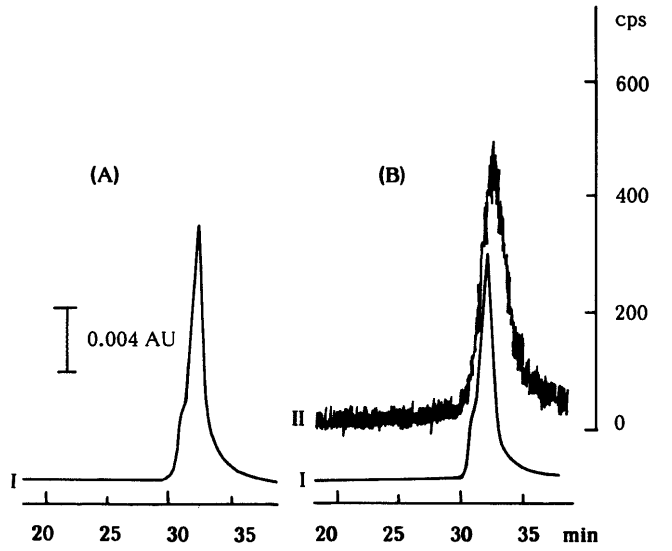

Fig. 4. Chromatograms of HDA (A) and ^{99m}Tc-HDA (B)
Chromatographic conditions are the same as those in Fig. 1.

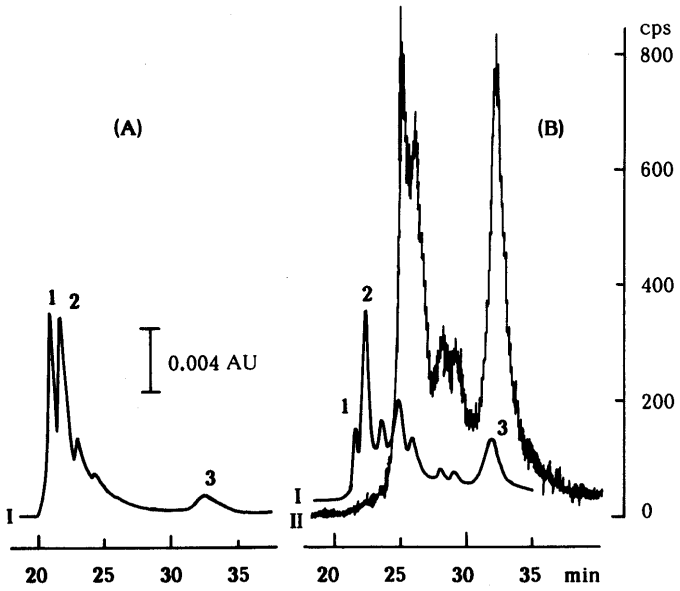

Fig. 5. Chromatograms of HSA (A) and (^{99}Tc+^{99m}Tc) HSA (B)
Number of peaks and chromatographic conditions are the same as those in Fig. 1.

due to the sulfhydryl group in it. Furthermore, the conformational change may also affect the reactivity. Since HNA was labeled with ^{99m}Tc, the following is considered: (1) HNA also possesses some binding sites which can be bound to ^{99m}Tc, (2) ^{99m}Tc preferentially binds to the inherent sulfhydryl groups or those produced by the reduction. Reactivity of HDA for ^{99m}Tc can be similarly explained.

To elucidate the effect of HMA in the labeling of HSA with ^{99m}Tc, HSA was labeled with ^{99}Tc. Since ^{99}Tc is an β emitter with a long half-life (2.14×10^5 y.), a stoichiometric scale is applicable to this experimental system. The reaction of ^{99}Tc and HSA can be monitored by UV spectrophotometrically. The amount of R-Sn used was 3 times that of the tracer scale and 95% labeling efficiency was obtained.

The chromatogram of ^{99}Tc-HSA in Fig. 5 shows a decreased HMA peak and no change in that of HNA. This implies a selective, high reactivity of HMA for technetium. In addition, decrease of the HMA peak was accompanied by the formation of four new peaks (25, 26, 28 and 29 min) which were not shown in HSA and an increase the polymerized albumin peak which eluted at 32 min. The total increase in peak area of these five peaks coincided to the amount of decrease in HMA peak; therefore, all were complexes of mercaptalbumin and Tc. They also corresponded to the radiochromatographic peaks shown in Figs. 1B and 3B. It is known that reduction of TcO_4^- results in several Tc oxidation states. The binding of Tc having different oxidation states[11,25] may bring about a change in the albumin conformations which would elute at various retention times from the 4VP column. Another possibility is that the labeled compounds contain different numbers of Tc atoms per albumin molecule, however, we were unable to confirm this by chromatographic methods. When HMA was labeled with ^{99}Tc, a similar chromatographic change to that of HSA was obtained. Although commercially available HSA is mainly composed of HMA and HNA, it is thought that technetium preferentially reacts to HMA.

It is concluded that the ion-exchange mode HPLC using the 4VP column presented here would be applicable as an analytical method for ^{99m}Tc-HSA. This method made possible clarification of the role of mercaptalbumin in the reaction of ^{99m}Tc and HSA, and may also offer more useful information about the quality of ^{99m}Tc-HSA.

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