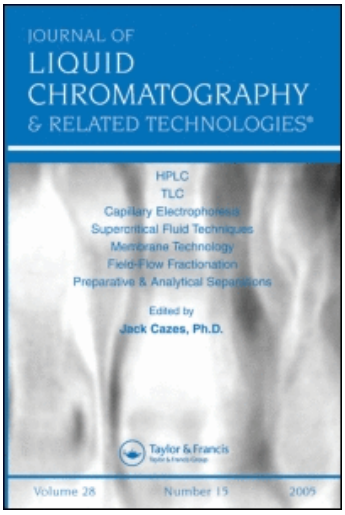

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APPLICATION OF AN N-METHYLPYRIDINIUM POLYMER COLUMN TO CHROMATOGRAPHIC INVESTIGATION OF HUMAN SERUM ALBUMIN-SODIUM AUROTHIOMALATE COMPLEX

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ABSTRACT

A column containing N-methylpyridinium polymer was used in the chromatographic investigation of complex of human serum albumin and sodium aurothiomalate. The serum albumin-sodium aurothiomalate complex was separated from the components of serum albumin. The peak height of the complex increased with increasing the incubation time of albumin and sodium aurothiomalate, with a concomitant decrease of mercaptalbumin peak height. The nature of the mercaptalbumin participation in this complex formation was elucidated. The formation of the complex was observed in vitro, but not in the serum of a patient with rheumatoid arthritis receiving sodium aurothiomalate therapy.

INTRODUCTION

Sodium aurothiomalate (AuSTm), a therapeutic agent for rheumatoid arthritis (RA), binds with high affinity to serum albumin. There have been reports concerning binding in vivo (1,2)

and in vitro (3-6) and several have suggested the participation of the sulfhydryl group of albumin in the binding (5-7). Shaw et al. also examined the structure of bovine serum albumin and AuSTm complexes using various physicochemical techniques (8). They indicated that gold(I) was coordinated with two sulfur atoms and assumed that the high affinity binding site on albumin for AuSTm was probably the sulfhydryl group cys-34 in mercaptalbumin. However, this has yet to be confirmed.

Serum albumins are heterogeneous with respect to sulfhydryl content and consist of mercaptalbumin and non-mercaptalbumin. The N-methylpyridinium polymer (4VP-Me) column developed for high performance liquid chromatography (HPLC) by Sugii et al. (9) can resolve these albumin components. Using this column to study the binding of AuSTm to serum albumin should clarify the role of mercaptalbumin.

In this study, the reactivity of AuSTm with human serum albumin (HSA) was investigated by HPLC using a 4VP-Me column. Furthermore, the serum from a RA patient receiving AuSTm therapy was analyzed using this column and the presence of albumin-AuSTm complex in the serum is discussed.

EXPERIMENTAL

Reagents

Fraction V human serum albumin was supplied by the Chemo-Serotherapeutic Research Institute (Kumamoto, Japan) and sodium

aurothiomalate (AuSTm) from Shionogi Pharm. Ltd. (Osaka, Japan). Thiomaleic acid (Tm) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), and L-cysteine from Nacalai Tesque, Inc. (Kyoto, Japan). Standard gold solution(5.1×10^{-3} M) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Human mercaptalbumin (HMA) and human non-mercaptalbumin conjugated with cysteine (HNA) were prepared by the method of Sogami et al. (10).

Apparatus

The HPLC system was constructed with a Hitachi (Tokyo, Japan) 655A-11 pump equipped with a Hitachi L-5000 low-pressure gradient programmer and a Hitachi 655A variable wave length UV monitor.

The gold concentration was determined using a Hitachi Z-8000 Zeeman-effect flameless atomic absorption spectrophotometer.

Chromatography

Samples were chromatographed on N-methylpyridinium polymer cross-linked with ethylene glycol dimethacrylate (4VP-Me column, 10-15 μm, 250 X 4 mm I.D.) (9). Samples were eluted with a 30-min linear gradient from 0 to 0.5 M sodium acetate in 0.05 M Tris-CH_3COOH buffer (pH 7.0) at a flow rate of 0.5 ml/min. Samples were eluted at room temperature and detected at 280 nm.

Unless stated otherwise, albumins ($3x10^{-4}$ M) were incubated with AuSTm ($3x10^{-4}$ M) in 0.05 M phosphate buffer containing 0.1 M NaCl (pH 7.4) for a defined duration at 37 °C. Aliquots (15 µl) were then injected into the chromatographic system and the eluate was fractionated. Gold was determined in the fractions by flameless atomic absorption spectrophotometry. Sera obtained from healthy volunteers (0.1 ml) were incubated with the same volume of AuSTm solution ($7.6x10^{-4}$ M) and were chromatographed in a similar manner as above.

RA patient serum (15 µl) was applied to the column and the eluate was fractionated (fraction volume 0.5 ml). Every 5 fractions were pooled and concentrated to one twenty-fifth of the original volume (Mole cut II, Millipore-UFPI LGC, Nihon Millipore Limited, Tokyo, Japan). The gold concentration in the concentrated fractions was determined.

HPLC using TSK-G3000SWXL column (Tosoh Co.Ltd., Tokyo, Japan) was performed by isocratic elution with 0.1 M phosphate buffer containing 0.3 M NaCl (pH 7.0) at a flow rate of 0.5 ml/min.

RESULTS AND DISCUSSION

The 4VP-Me column can resolve albumin components such as mercaptalbumin and non-mercaptalbumins. Non-mercaptalbumins usually consist of two mixed disulfides, i.e. with cysteine and glutathione. However, we previously reported that the non-

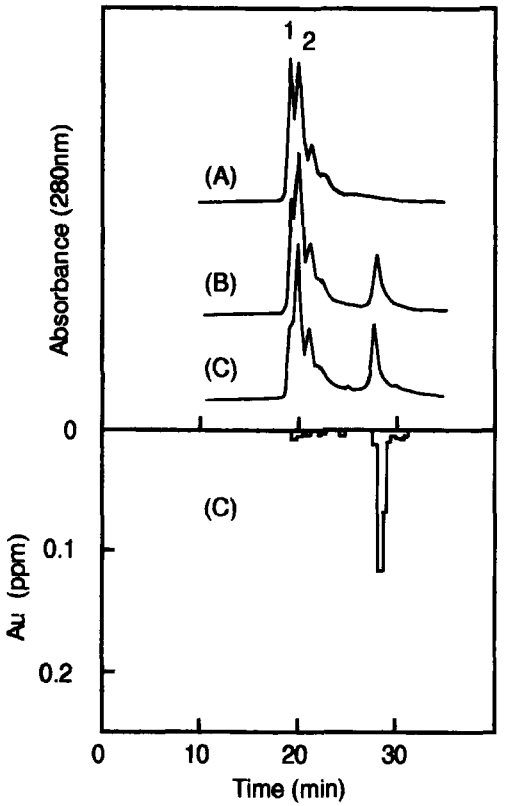

FIGURE 1. Chromatograms of HSA incubated with AuSTm and histogram of the gold concentration in the eluent.
Incubation time:(A)0hr, (B)6hr, (C)12hr.
Peaks:(1)mercaptalbumin, (2)non-mercaptalbumin

mercaptalbumins in serum albumin were mainly mixed disulfide with cysteine (11). The chromatogram in Fig.1(A) shows a separation profile typical of the HSA preparation. The HSA chromatogram showed three main peaks of mercaptalbumin (HMA), non-mercaptalbumin of mixed disulfide with cysteine (HNA) and a highly oxidized form of HMA.

In the chromatograms of HSA incubated with AuSTm, a peak containing gold was observed at 28 min. The HMA peak height decreased with increasing incubation time, however, the HNA peak was scarcely affected. On the other hand, when only the AuSTm was injected into the column, it was strongly retained. This fact shows the AuSTm is negatively charged under the conditions used and adsorbed onto the anion-exchange 4VP-Me column. Gerber (7) stated that no evidence existed for the presence of free thiomalate in solutions containing AuSTm and serum albumin which were incubated for 72 hr. Therefore, the dissociation of AuSTm to gold and thiomaleic acid during the incubation may be discounted. However, HSA was incubated with hydrochloroauric acid or thiomaleic acid under the same conditions as AuSTm to confirm this. In the chromatogram of HSA incubated with hydrochloroauric acid, no peak was observed at 28 min in spite of the mercaptalbumin decrease. In the reaction of HSA with thiomaleic acid, a peak appeared at 25 min, however this peak did not contain gold. These results support the conjecture that the peak (t_R=28 min) that appeared after incubating HSA and AuSTm corresponds to the HSA-AuSTm complex.

We subsequently attempted to elucidate the contribution of mercaptalbumin in the formation of the AuSTm complex, using HMA and HNA prepared separately. Chromatograms of HMA and HNA incubated with AuSTm are shown in Figs. 2 and 3, respectively. Only HMA reacted with AuSTm and the peak containing gold appeared

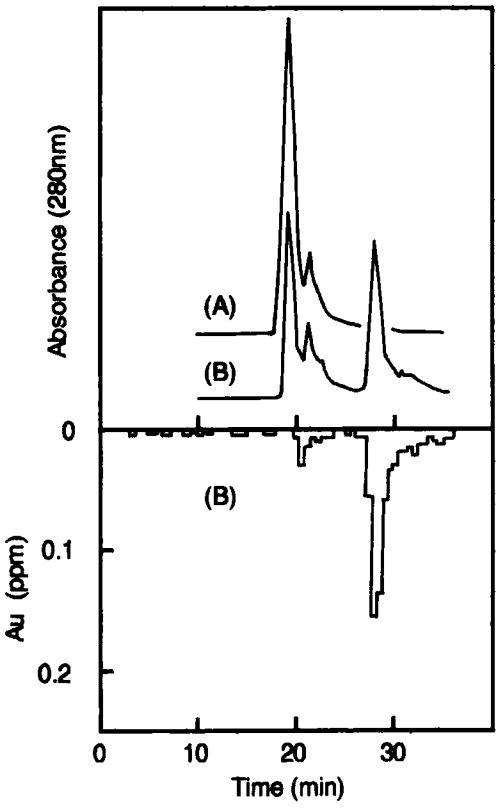

FIGURE 2. Chromatograms of HMA incubated with AuSTm and histogram of the gold concentration in the eluent.
Incubation time:(A)0hr, (B)12hr.

at 28 min. Chromatographic effects and gold incorporation were scarcely observed for HNA incubated with AuSTm.

Isab and Sadler (12) demonstrated an inverse correlation between the pKa value of the sulfhydryl group(pK_{SH}) and gold affinity. They mentioned that thiol having a lower pKa value bound gold with higher affinity. Shaw (13) described that the cys-34 in

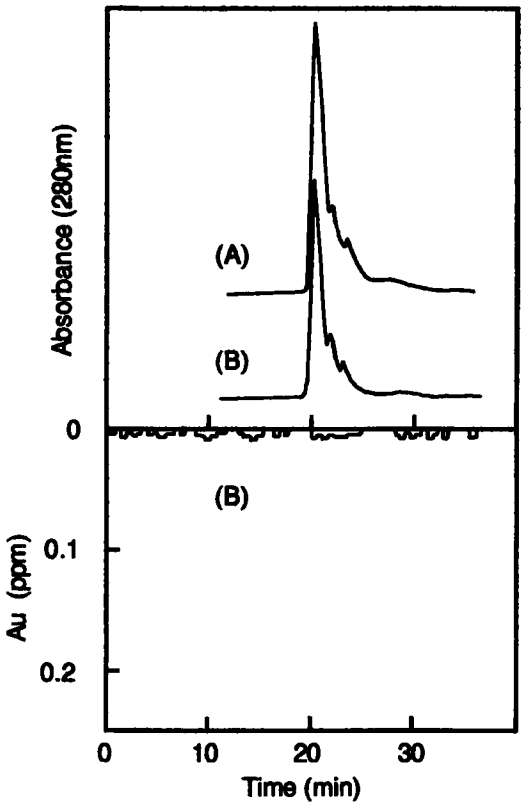

FIGURE 3. Chromatograms of HNA incubated with AuSTm and histogram of the gold concentration in the eluent.
Incubation time:(A)0hr, (B)12hr.

albumin with a low pK_{SH}(<5) (14), compared with that of cysteine(8.5) or glutathione(8.9), should have high affinity for gold. Our chromatographic data also support this idea. In addition, Shaw also mentioned the possibility of a weak binding site other than cys-34. The weak binding site, however, was not confirmed by our chromatographic data with HNA.

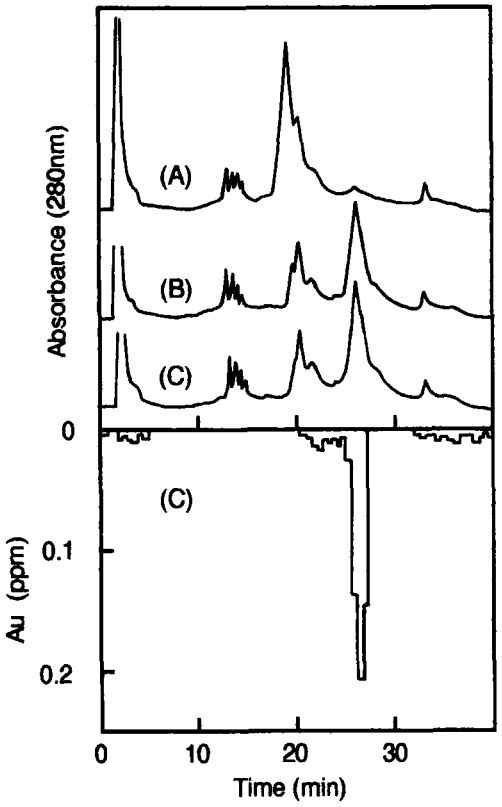

FIGURE 4. Chromatograms of normal serum incubated with AuSTm and histogram of the gold concentration in the eluent.
Incubation time:(A)0hr, (B)6hr, (C)12hr.

The formation of the peak at 28 min was studied in more detail. When various molar ratios of HSA to AuSTm (1:1,1:4,1:10) were incubated under the same conditions, the same chromatogram was obtained. And the molar ratio of albumin to gold in the peak fraction at 28 min was about 1:1. Excess AuSTm in the reaction system may produce albumin-$(AuSTm)_n$ complexes (n>1) as shown by

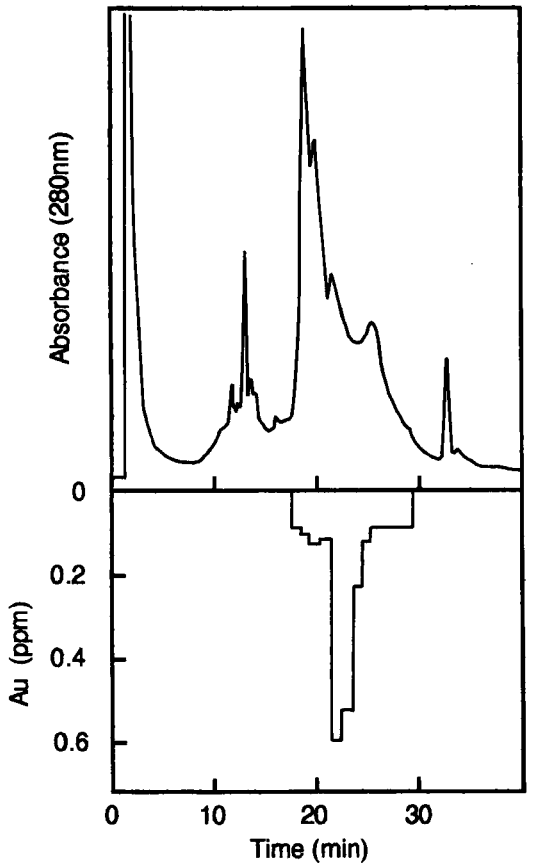

FIGURE 5. Chromatogram of rheumatoid patient serum and histogram of the gold concentration in the eluent.

Shaw et al. (8). However, it seems likely that AuSTm bound weakly on the albumin releases from the complex in the ion-exchange chromatographic process. The retention time of albumin-AuSTm complex was longer than that of albumin. That HSA-AuSTm eluted more slowly than HSA is explained on the basis of the change in

negative charge density at the outer surface of the complex molecule. Although the contribution of the carboxyl group of AuSTm to the charge is not negligible, a conformational change may be important.

Jellum et al. (15) reported that AuSTm administered by intramuscular injection decomposed to gold and thiomalate moieties in vivo. This suggests a low probability of albumin-AuSTm complex being in the blood. To confirm this, the behavior of AuSTm in serum was further examined. First, AuSTm was incubated with serum from a healthy volunteer. As shown in Fig.4, the peak of the albumin-AuSTm complex appeared at 28 min. Next, the serum of a patient with RA receiving AuSTm was chromatographed 3 hr after the final administration (Fig.5). The peak corresponding to albumin-AuSTm (t_R=28 min) was not observed. However, a gold rich protein fraction appeared at 22 min. This fraction was analyzed by size exclusion chromatography on a TSK gel G3000SWXL column. Gold was found in the peak corresponding to albumin monomer. Therefore, the compound contained in the gold rich fraction would be an albumin coordinated with a small molecular gold compound other than AuSTm. The in vivo "dissociation" of thiomalate from AuSTm should be considered. Though the ligand exchange reaction that occurred with albumin-AuSTm in serum is considered as suggested by Shaw et al.(16), gold containing metabolites of AuSTm may also bind to albumin.

It is concluded that the 4VP-Me column presented here was useful to study of the reaction of AuSTm with albumin and the high

reactivity of mercaptalbumin with AuSTm was elucidated. Furthermore, the consequence of the lack of albumin-AuSTm complex in the serum from a patient with RA may shed light on the fate of AuSTm in vivo.

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