AN INVESTIGATION OF THE EFFECT OF THE QUANTITY OF STANNOUS ION ON THE QUALITY OF TECHNETIUM-99m LABELLED ALBUMIN

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SUMMARY

The importance of maintaining a low ratio of stannous ion to albumin molecules in order to obtain a high quality technetium-99m labelled albumin is demonstrated. It is further shown that the direct preparation of technetium-99m albumin by reduction of the technetium-99mm pertechnetate with stannous ion inevitably leads to the contamination of the product with a certain amount of tin colloid which is also labelled with technetium-99m. It is demonstrated that this can be avoided by utilizing labelling techniques involving the initial formation of other technetium chelates which are less stable than the albumin complex under certain conditions, adding the albumin to that preparation, adjusting the conditions and thus allowing the albumin to become labelled with technetium-99m via an exchange with the original chelating agent.

Key Words: Technetium-99m, Albumin, Quality, Stannous

INTRODUCTION

Technetium-99m human serum albumin is widely used for blood pool scanning, both cardiac and placenta. The increasing use of cardiac studies result in a need for a high radioactive concentration in the blood over a period of 30 minutes or more. This can easily be done simply by injecting the patient with large doses, however if a significant fraction is rapidly removed from the blood due to either impurities in the preparation or the formation of dimer or higher aggregates [1], then the patient is being exposed to unnecessary radiation, and, furthermore, this removed fraction may contribute to the general background and thus reduces the quality of the study. That the preparation of technetium-99m human serum albumin is of some interest is demonstrated by several papers appearing recently in the

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Journal [1, 2]. This paper will study the effects of a number of variables on the quality and composition of technetium-99m human serum albumin in an attempt to indicate how a "good" monomeric technetium-99m labelled albumin with good in vitro stability might be prepared.

METHODS AND MATERIALS

1) Preparation of the ^{99m}Tc Human Serum Albumin

The ^{99m}Tc H.S.A. used throughout the major part of this study was prepared using our electrolytic tin labelling technique. An electrolytic vial is prepared by inserting a pair of tin wire electrodes through the rubber septum of a 10 ml multidose vial. The desired quantity of 25% salt poor human serum albumin (Connaught) is injected into the vial with 4 ml of ^{99m}Tc pertechnetate solution and 2.0 ml of decinormal hydrochloric acid. The vial is then placed in an ultrasonic bath and the electrodes connected to a constant current power supply, which is adjusted to the desired current and then turned on for an appropriate length of time to generate the selected quantity of stannous ion. The electrodes are immediately removed from the vial, 1 ml of 10% dextrose and 2 ml of sodium acetate buffer, containing 13.5% sodium acetate trihydrate w/v adjusted to pH 5.6 with 2N acetic acid, are added to the vial. The preparation is then allowed to stand for an hour before being sterilized by millipore filtration.

In preparations in which tin-113 was used as a tracer it was added in the form of stannous chloride in hydrochloric acid solution immediately prior to electrolysis. The quantity of tin added in this manner was small since the specific activity was 15 mCi/mgm and only 200 uCi (ie. 13 ugm) of tracer was used in each preparation.

In those studies where repurified human serum albumin was used the repurification was carried out by gel chromatography on a 60 x 1.5 cm column of Sephadex G200 which was eluted with physiological saline. The eluant was collected in 40 drop (approximately 1.2 ml) fractions. Alternate fractions were assayed for albumin content of the remaining samples was then estimated from the average value of their immediate neighbours and a volume corresponding

to 6.25 mgm (9.3×10^{-5} moles) of albumin used in a standard technetium-99m human serum albumin preparation using a 2 mA current for 1 minute.

The indirect labelling technique of Wong et al [3] which involves the formation of technetium-99m citrate which is then used to label the albumin with technetium-99m was modified by substituting electrolytic generation of the same quantity of stannous ion for the stannous chloride solution and the technetium-99m prepared in this way compared with that prepared by the normal direct route.

One technetium-99m albumin preparation was prepared by a ligand exchange technique using technetium-99m gluconate as the starting chelate. Calcium gluconate (2 ml of a 10% w/v solution) and technetium-99m pertechnetate and saline (10 ml) were added to a multidose vial into the cap of which two tin wires had been inserted. The vial was placed in an ultrasonic bath and a 0.1 mA current passed between the tin electrodes for 10 minutes. The pH was adjust to 2.5 with 1N hydrochloric acid, the preparation allowed to sit for half an hour and then filtered before 0.1 ml of 25% w/v human serum albumin was added. This preparation was then incubated at 37°C for 2½ hours being being analyzed.

2) Analysis of ^{99m}Tc Albumin Preparations

The quantity of free pertechnetate in the various technetium albumin preparations was analyzed using thin layer silica gel chromatograms developed in methyl ethyl ketone. Gel chromatography of the preparations was performed on a 60 x 0.9 cm Sephadex G200 column (Pharmacia Fine Chemicals) with 0.2 ml of the technetium albumin preparations being applied to the top of the column and eluted with physiological saline. The eluant was collected in 20 drop (approximately 0.6 ml) fractions. This column was used to examine the quality of the labelled protein species including the question of whether there was more than one polymeric species, ie was both a monomer and a dimer present. All ^{99m}Tc human serum albumin preparations were sterilized by millipore filtration through 0.22 micron filters, however early evidence, supported by Pettit et al [2], suggested the presence of colloidal material smaller than 0.22 microns. To examine this aspect a series of preparations

at a variety of tin to albumin ratios were filtered through nucleopore 0.05 micron filters. One ml of the ^{99m}Tc albumin preparation was taken and passed through the filter followed by a 5 ml saline wash. A 60 lb/sq.in. press was used to force all the liquid through the filter. The radioactivity in the filtrate and that retained on the filter was then evaluated in a radioisotope calibrator.

In those preparations in which tracer quantities of ^{113}Sn were incorporated all samples were stored for two days after the technetium-99m radioactivity had been counted to allow technetium-99m to decay and also to allow for the decay of the ^{113m}In daughter of ^{113}Sn which was present in the material prior to separation, thus at the time of counting any ^{113m}In present would have come from the ^{113}Sn after separation and would be in isotopic equilibrium with it. In this way the counting of the 393 KeV gamma ray of ^{113m}In is representative of the quantity of ^{113}Sn present in the sample.

Thin layer silica gel chromatography strips were allowed to soak in a 5% solution of human serum albumin for half an hour and then air dried and stored in the refrigerator until required. Silica gel chromatograms treated in this way do not show the loading artifact referred to by Lin et al (4) and when developed in saline only colloidal material or hydrolyzed reduced technetium remains at the origin. Thin layer silica gel chromatograms treated in this manner and developed in saline were utilized in the analysis of all technetium-99m albumin preparations.

RESULTS

1) Membrane Filtration Studies

The results of this study in which three different concentrations of human serum albumin were studied in conjunction with a variety of stannous ion concentrations are shown in figure 1. These results clearly show that, irrespective of the concentration of albumin or stannous ion, the labelled species at stannous to albumin molecular ratios of greater than 35 to 1 has a diameter of more than 0.05 microns, while below that ratio virtually all the labelled product is smaller than 0.05 microns.

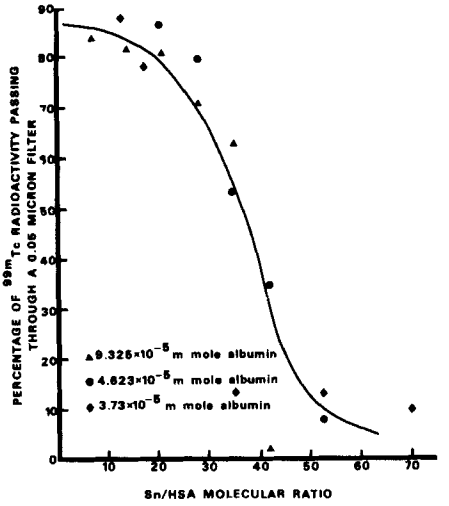

FIG. 1 The percentage of ^{99m}Tc radioactivity in ^{99m}Tc albumin which passes through a 0.05 micron filter as a function of the tin to albumin molar ratio.

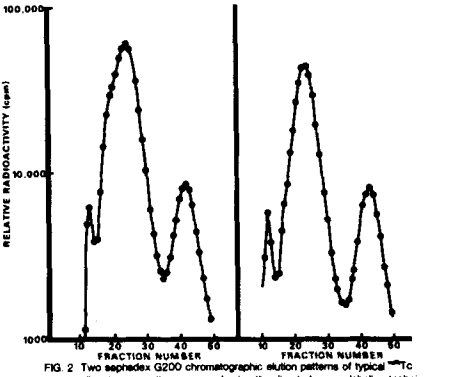

FIG. 2 Two sephadex G200 chromatographic elution patterns of typical ^{99m}Tc albumin preparations prepared using the direct stannous labeling technique.

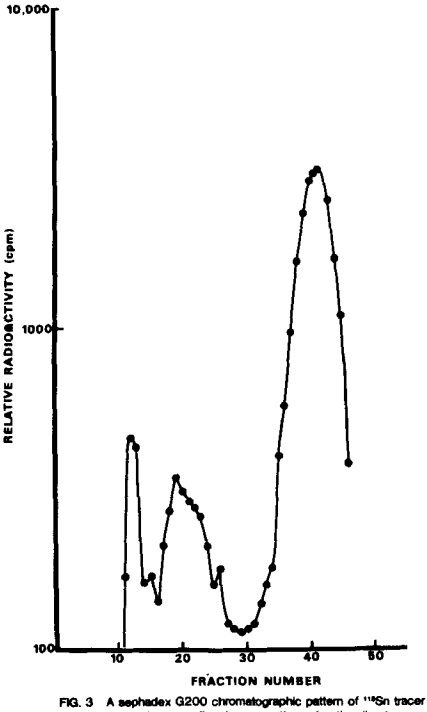

FIG. 3 A sephadex G200 chromatographic pattern of ^{113}Sn tracer in a technetium albumin preparation using the direct stannous labeling technique.

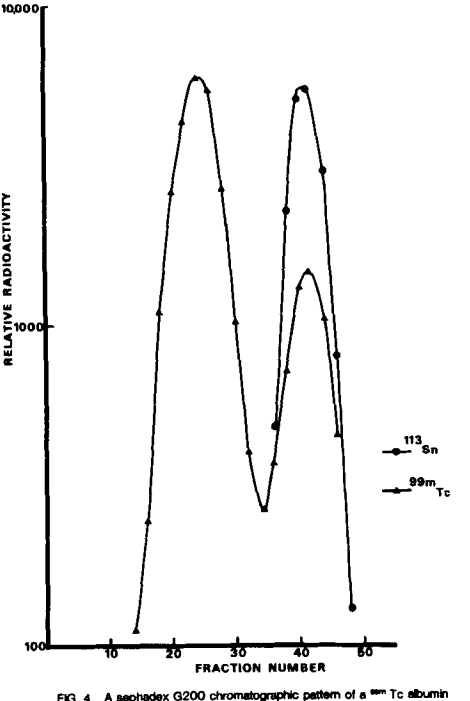

FIG. 4 A sephadex G200 chromatographic pattern of a ^{99m}Tc albumin preparation prepared by the isotope exchange technique with technetium gluconate. The ^{99m}Tc and the ^{113}Sn patterns are both shown and indicate that the tin remains with the gluconate.

2) Sephadex G200 Studies

Two typical curves obtained from this column are shown in Figure 2. There are several observations which can be made from these curves. Obviously the product is not a single pure labelled species, in fact four distinct radioactive species are easily identified, these are the peak at fraction #12(A), the main peak itself at fraction #24(C), the shoulder on this main peak at around fraction #19(B) and the final peak at fraction #43(D). The relative proportions of these species were found to vary with the relative proportions of albumin and stannous ion and with the concentration of the preparation. The data obtained from these studies is recorded in Table 1.

TABLE 1

Components of Technetium-99m Albumin Labelled by the Direct Stannous Ion Technique

HSA (m moles)	Sn (m moles)	Sn/HSA	A	B	C	D
9.3×10^{-5}	1.58×10^{-4}	1.69	2.08	6.47	74.63	16.80
9.3×10^{-5}	3.15×10^{-4}	3.39	2.13	2.53	79.04	16.25
9.3×10^{-5}	6.30×10^{-4}	6.77	2.24	1.02	80.56	16.19
9.3×10^{-5}	1.26×10^{-3}	13.55	5.16	9.14	73.44	12.26
9.3×10^{-5}	2.52×10^{-3}	27.10	20.04	16.17	54.62	9.17
1.86×10^{-4}	3.15×10^{-4}	1.69	2.87	3.00	85.57	8.56
1.86×10^{-4}	6.30×10^{-4}	3.39	2.02	2.69	87.40	7.90
1.86×10^{-4}	1.26×10^{-3}	6.77	6.60	2.99	82.00	8.42
1.86×10^{-4}	1.89×10^{-3}	10.16	7.88	2.92	78.77	8.40
7.44×10^{-4}	6.3×10^{-4}	0.85	3.80	15.77	71.55	8.86
3.72×10^{-4}	6.3×10^{-4}	1.69	2.89	8.88	80.03	8.21
1.86×10^{-4}	6.3×10^{-4}	3.39	2.02	2.69	87.40	7.90
9.3×10^{-5}	6.3×10^{-4}	6.77	2.24	1.02	80.56	16.19
3.72×10^{-5}	6.3×10^{-4}	16.94	12.40	0	72.57	15.03

where A is the peak centered at fraction #12
B is the shoulder at fraction #19
C is the major peak centered at fraction #24
D is the peak centered at fraction #43

Note that the area under the shoulder at #19 is estimated assuming symmetry at the major peak at fraction #24, an assumption which is supported by the symmetry of the major peak at this point obtained in the preparation using exchange labelling from technetium gluconate (see later). Figure 3 shows a typical distribution of ^{113}Sn after Sephadex G200 chromatography of a technetium-99m preparation prepared by the direct labelling technique.

The results of the purification of the commercial supply of human serum albumin showed a distribution which was approximately symmetrical and gave no indication of any significant shoulder corresponding to fraction #19 in the labelled studies, however when samples from various fractions around the peak were labelled with technetium the pattern observed was identical to that obtained with the commercial albumin.

The Sephadex G200 gel chromatography of the technetium-99m albumin prepared by the modified indirect method of Wong et al (3) showed significant technetium-99m components in each of the fractions identified in the gel chromatography of the technetium albumin preparations obtained by the direct method and in terms of these contaminants this indirect method showed no advantages over the direct method.

The results of the analysis of the technetium-99m albumin prepared indirectly from technetium-99m gluconate are shown in figure 4. The preparation showed no signs of colloidal tin at void volumes. The area of fraction #43 showed a significant peak however this is the area at which technetium gluconate is eluted from the Sephadex G200 column and this peak is believed to represent technetium-99m gluconate since silica gel thin layer chromatograms pretreated with human serum albumin and developed in saline showed no sign of any colloidal technetium. Similarly thin layer silica gel chromatograms developed in methyl ethyl ketone showed no sign of free pertechnetate. This preparation also contained ^{113}Sn as a tracer and figure 4 shows that the tin was associated with the fraction eluted around fraction #43, ie it appears that all the tin remains associated with the gluconate.

DISCUSSION

The membrance filtration studies clearly indicate that if the ratio of stannous ion to albumin molecules becomes too high, ie greater than about 20, then the product was no longer a simple technetium labelled albumin but a species that had a diameter larger than 0.05 microns. If such aggregates were formed in such large proportions when the stannous to albumin ratio reached this level then clearly smaller aggregates are probably formed at lower stannous to albumin ratios. Thus gel filtration studies on a series of preparations at lower stannous to albumin ratios were instituted. The elution pattern obtained from Sephadex G200 column (figure 2) show that there are basically four components which appear in varying proportions. These are the radioactivity appearing at void volume (fraction #12); the radioactivity around fraction #19 which appears as a shoulder on the main peak at fraction #24; and finally the radioactivity around fraction #43. In addition free pertechnetate was shown to migrate at fraction #47 on the Sephadex G200 column and any preparation showing any sign of a peak at this value was discarded. The peaks at void volume and at fraction #43 appear to be the same peaks as reported by Pettit et al [2], i.e. they appear to be tin colloid peaks. If the percentage of the radioactivity (^{99m}Tc) present in these is compared with the percentage of the ^{99m}Tc found to be colloidal as determined by the albumin treated silica gel thin layer chromatograms, Table 2, good agreement is obtained. Tables I and 2 show that the colloidal tin peaks, ie. the peaks occuring at fractions #12 and #43 account for at least 10% of the ^{99m}Tc radioactivity and that this quantity is at a minimum when the tin to albumin ratio is 3.39. In the case of the series at low albumin concentration the minimum value is observed over a wide tin to albumin ratio range but it is around 18% suggesting that the low albumin concentration allows the formation of a greater proportion of labelled colloid. This could be due to more colloid formation or simply the lower amount of albumin competing less favourably for the technetium. In those cases where the tin to albumin ratio is lower than 3.39 there is a slight increase in the sum of these two peaks

TABLE 2

^{99m}Tc Colloid in ^{99m}Tc Albumin Preparations

HSA (m moles)	Sn (m moles)	Sn/HSA	^{99m}Tc Colloid by TLC	A & D Peaks
9.3×10^{-5}	1.58×10^{-4}	1.69	18.5	18.9
9.3×10^{-5}	3.15×10^{-4}	3.39	17.8	18.4
9.3×10^{-5}	6.30×10^{-4}	6.77	18.1	18.4
9.3×10^{-5}	1.26×10^{-3}	13.55	18.2	17.4
9.3×10^{-5}	2.52×10^{-3}	27.10	30.1	29.2
1.86×10^{-4}	3.15×10^{-4}	1.69	13.3	11.4
1.86×10^{-4}	6.30×10^{-4}	3.39	9.8	9.9
1.86×10^{-4}	1.26×10^{-4}	6.77	15.6	15.0
1.86×10^{-4}	1.89×10^{-4}	10.16	17.8	16.3
7.44×10^{-4}	6.30×10^{-4}	0.85	12.0	12.7
3.72×10^{-4}	6.30×10^{-4}	1.69	9.5	10.1
1.86×10^{-4}	6.30×10^{-4}	3.39	9.8	9.9
9.3×10^{-5}	6.30×10^{-4}	6.77	18.1	18.4
3.72×10^{-5}	6.30×10^{-4}	16.94	30.5	27.4

however the prime contaminant appears to be very significant in the shoulder around fraction #19 suggesting that this shoulder could relate to some sort of albumin dimer. The ^{113}Sn distribution curve shown in figure 3 clearly indicates that tin is associated with this entity. In those cases where the tin to albumin is greater than 3.39 there is a significant increase in the sum of peaks A and D with peak A generally showing the greater increase suggesting that the higher concentration of tin leads to more colloid formation. It was also noted that at the low albumin concentration the shoulder at fraction #19 also increased significantly at higher tin to albumin ratios although this was not observed at other albumin concentrations. When tracer amounts of ^{113}Sn were introduced into the product at the beginning of the preparation in the form of stannous chloride in hydrochloric acid, the Sephadex G200 elution pattern obtained, figure 3, confirms that most of the ^{113}Sn was present in the peaks A and D. Significantly there was very little

^{113}Sn observed in the area of the major ^{99m}Tc peak at fraction #24 suggesting that the stannous ion does not associate with the albumin molecule in any simple manner. This fact probably explains why all albumin preparations prepared by a direct stannous labelling technique are apparently contaminated with colloidal tin labelled with technetium. This may seem at odds with the initial observation that the size of the particle formed exceeded 0.05 micron in diameter only when the stannous to albumin exceeded 35 to 1, however it should be remembered that albumin acts as a protective agent to colloids and it is probably exhibiting this property in helping prevent the size of the tin colloid from growing, thus the size of the tin colloid formed is to an extent dependent on the ratio of tin to albumin.

The fact that the technetium albumin prepared by the indirect method of Wong et al (3) also showed sizeable amounts of technetium radioactivity associated with peaks A and D may suggest that as the pH is raised not only does the bond between the citrate and the technetium become unstable, but also the complex of the tin and the citrate also becomes unstable allowing the tin to hydrolyze and become colloidal, of course once there is colloidal tin present the technetium which is dissociating from the citrate may become bound either to the albumin or to the colloid.

The technetium-99m labelled albumin prepared via exchange labelling from technetium-99m gluconate showed no sign of a tin colloid peak at fraction #12 nor any shoulder at fraction #19. The secondary peak at fraction #4 is still associated with the gluconate. It is also important to notice that all the ^{113}Sn is found in this region indicating that the tin is still firmly associated with the gluconate. Unfortunately there is still 18% of the technetium-99m associated with the gluconate so that without further purification the albumin prepared in this way is not pure enough for clinical use, furthermore the separation of the gluconate from the albumin would remove all the stannous ion and result in a technetium albumin product which would not have a very good in vitro stability unless some other anti-oxidant were added eg. ascorbic acid (5). Variation of the

conditions used during technetium exchange resulted in some improvement i.e. by lowering the pH to 1 during the exchange period and re-adjusting it to 4.5 at the end of the exchange and reducing the amount of gluconate in the preparation it was possible to obtain a stable preparation with 90% of the technetium-99m in the form of technetium albumin with only one hour being allowed for the exchange. Thus the preparation time was reduced to a more acceptable one and half hours and the impurity, which was totally technetium gluconate, reduced to 10%. This however is hardly better than that obtained by direct stannous labelling so that changing the preparation procedure merely changes the impurity substituting the liver for the kidney in terms of target organ for the impurity. Thus while it appears that this approach is likely to yield a technetium-99m albumin which is not contaminated by colloidal tin or any dimer type compounds the lack of complete exchange from the gluconate suggests that alternate chelating agent should be found to constitute the initial technetium product.

CONCLUSIONS

These results lead to the conclusion that a high quality technetium-99m labelled human serum albumin cannot be prepared by a direct technetium-99m labelling technique using the stannous ion as the reducing agent for the pertechnetate since this inevitably gives rise to labelled tin colloid. The appearance of the tin colloid in the preparation is probably due to the fact that the stannous ion does not appear to form a stable chelate with a single albumin molecule. The most promising approach to the preparation of a high quality technetium-99m labelled human serum albumin appears to be via an exchange labelling technique where the initial chelating agent will serve to solubilize the stannous ion and prevent its hydrolysis while being a relatively weak chelating agent for the reduced technetium and therefore allowing its virtually complete transfer to the albumin molecules. To achieve this the chelating agent should form a stronger chelate with the stannous ion than citrate does, but a weaker chelate with technetium than gluconate.

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