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Labelling of Human Serum Albumin with ^{99m}Tc Using Sn(II) Citrate.

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Summary

Labelling of human serum albumin (HSA) with ^{99m}Tc, at neutral pH, using Sn(II) citrate as reducing agent is described. At pH 7.4, more than 98% binding efficiency for ^{99m}Tc-HSA is achieved. ^{99m}Tc-Sn-citrate with high protein binding capacity is first formed as an intermediate complex and in the presence of albumin, a more stable ^{99m}Tc-HSA complex is formed. ^{99m}Tc-HSA complex is formed by ligand exchange and 30 min reaction time is sufficient for the complex formation of ^{99m}Tc-HSA. Biodistribution in normal mice at 30 min obtained for ^{99m}Tc-HSA prepared by Sn(II) citrate showed 30.3 % injected dose in blood compared to 32.9-34.5 % for ^{99m}Tc-HSA prepared by Sn(II)Cl_2. The presence of citrate stabilizes Sn(II) ions towards oxidation and hydrolysis. The addition of ascorbic acid did not increase the percent yield of ^{99m}Tc-HSA and has adverse effect on its biological distribution.

Key Words :

Technetium-99m-human serum albumin, Sn(II) citrate, labelling, trichloroacetic acid protein precipitation, stability, ascorbic acid, drug biodistribution.

Introduction

Labelling of HSA with technetium-99m was first attempted in the early 1960s. Harper et al. [1] and Richards et al. [2] demonstrated that ascorbate or ascorbate plus Fe(III) could be

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used as reducing agents for technetium in this labelling procedure.

Electrolytic reduction of technetium has also been used in the preparation of ^{99m}Tc-HSA, first using zirconium electrodes [3-5], and later using tin wire electrodes [1,6,7]. The disadvantages of these methods include multiple pH adjustment, contamination of the labelled product with electrode materials and incomplete reduction of the pertechnetate.

More efficient labelling of HSA can be achieved by using Sn(II) as a reducing agent [8,9]. Various procedures have been developed for accomplishing this and a number of stannous reagent kits, which provide varying degrees of success, are commercially available at the present time. Pertechnetate usually does not persist as an impurity when using Sn(II) reduction method. However, colloidal technetium is often present in significant amounts if reaction conditions are not carefully controlled [10,11]. The labelling process using Sn(II) requires a single low pH reduction of technetium with the HSA present . This low pH is not suitable for proteins due to probable denaturation.

Stannous citrate is used in this study to minimize albumin denaturation since the labelling is carried out at neutral pH and also to minimize the possible formation of technetium-Sn-colloid due to the presence of Sn(II) as citrate complex. The different factors studied include reaction time, Sn(II) citrate content, HSA content, pH, biological distribution and stability. Finally, the effect of ascorbic acid on the labelling yield and biological distribution of Tc-HSA is also tested.

Experimental

Materials and Methods :

$^{99m}TcO_4^-$ was obtained from a sterile $^{99}Mo-^{99m}Tc$ generator Elutec TRE, Belgium. Human serum albumin (HSA) had been supplied by Serotherapeutisches Institut Wein Osterreich GmbH.

Stannous citrate was obtained from B.D.H. and was used without further purification. Sn(II) citrate solution was freshly prepared by adding 20 ml 2% trisodium citrate dihydrate in small portions to 100 mg Sn(II) citrate till clear solution was obtained. Heating on boiling water bath will help the dissolution of Sn(II) citrate. The final volume was adjusted to 50 ml and filtered through 0.22 μ Millipore filter. Each 1 ml of this solution contains 2 mg Sn(II) citrate, 8 mg trisodium citrate dihydrate and has pH 7.

Sn(II) determination :

Tin citrate (B.D.H.) was analyzed for its contents of stannous by iodometric titration using standard KIO_3 solution. The accuracy and reproducibility of this method for determining microamounts of Sn(II) had been confirmed [12]. The titration was carried out by introducing 0.35 ml 2N HCl, 0.05 ml starch solution and 1.0 ml tin citrate sample into 10 ml N_2 purged vial by means of 1.0 ml tuberculin syringes. The contents of the vial were mixed and titrated with standard KIO_3 solution in a tuberculin syringe until a permanent faint blue colour is formed. All solutions used were N_2 purged . The amount of Sn(II) content was determined from the following relation :

$$1.0 \text{ ml } 0.001N\ KIO_3 \cong 59.35\ \mu g\ Sn(II)$$

Labelling Procedure :

Reduction of $^{99m}TcO_4^-$ and albumin labelling were accomplished by adding 200 µl Sn(II) citrate solution to 0.1 ml 20 % HSA in 10 ml N_2 purged penicillin vial. The volume was completed to 2 ml with N_2 purged distilled water. The final pH was 7.4. 1 ml $^{99m}TcO_4^-$ was then added and the reaction mixture was incubated for 30 min at room temperature.

The binding efficiency of the labelled albumin is assessed by ascending paper radiochromatography in methyl ethyl ketone with Whatmann No. 1 paper.

Determination of the % labelling of ^{99m}Tc-HSA :

The purity and the % labelling of ^{99m}Tc-HSA were determined with trichloroacetie acid (TCA) protein precipitation method. One ml ^{99m}Tc-HSA was mixed with one ml 20% TCA and the precipitated ^{99m}Tc-HSA was separated by centrifugation. The activity of the supernatant and ^{99m}Tc-HSA precipitate were calculated by counting 0.2 ml of the supernatant and 0.2 ml of ^{99m}Tc-HSA solution used in the precipitation process. The % labelling was determined as follows :

$$\% \ ^{99m}\text{Tc-HSA} = \frac{\text{Activity of precipitated } ^{99m}\text{Tc-HSA}}{\text{Total activity of } ^{99m}\text{Tc-HSA solution}} \times 100$$

Biological Distribution :

0.1 ml of the ^{99m}Tc-HSA solution was injected in the tail vein of mice. A group of 3 were used for each determination. The mice were sacrificed 30 min after injection. Fresh blood was collected in a preweighed vial and counted. The different organs were removed, counted and compared to a standard dilution of the labelled radioalbumin. The average values of %

administered dose/organ are calculated after correction for radioactivity in the tail and total blood is assumed to be 7 % of the body weight.

Results and Discussion

$Sn(II)Cl_2$ is the most widely used reducing agent in the preparation of ^{99m}Tc-radiopharmaceuticals including ^{99m}Tc-HSA [13]. Because of the ease of its oxidation and hydrolysis , Sn(II) citrate is used in the present study. The presence of citrate stabilizes the Sn(II) ion towards oxidation and hydrolysis [14]. In addition, most of the published procedures[8 - 10] for HSA labelling with ^{99m}Tc using Sn(II) as reducing agent have been carried out at pH 1.5-2.5 to prevent tin colloid formation, but this pH is not suitable for proteins [15]. Labelling of albumin at neutral pH using Sn(II) citrate should minimize albumin denaturation.

Determination of Sn(II) :

The results obtained from the analysis of tin citrate (B.D.H.) solution showed that 60 % of its content is in the stannous form. The presence of oxidized tin in commercially available stannous salts is unavoidable [16] but the quantity found here is somewhat high compared to the measured quantity in some commercially available $SnCl_2.2\,H_2O$ which contain from 15 to 20% Sn(IV).

Determination of the Percent Labelling :

Several authors [10-12,17] have proposed assay methods for determining the quantities of labelled HSA in preparations. Only the amount of free $^{99m}TcO_4^-$ was observed in many of these methods and in addition ^{99m}Tc-HSA was not distinguished from reduced ^{99m}Tc-species that may be present. The presence of

^{99m}Tc-Sn-colloid in ^{99m}Tc-HSA preparation represents a real problem because it behaves similar to ^{99m}Tc-HSA in most of the analytical techniques[10,11,17]. The combination of analytical techniques together with bioassay is necessary for accurate estimation of the purity and quality of the labelled ^{99m}Tc-HSA.

The data presented in Table 1 show the average binding efficiency of ^{99m}Tc-HSA as assayed by paper radiochromatography (Whatmann No. 1) with MEK and saline, also by 20% TCA protein precipitation method and by Sephadex G-25 medium (pharmacia) gel column (1x30) chromatography eluted with 0.9% NaCl.

Table 1 : Binding efficiency of ^{99m}Tc-HSA as assayed by different analytical techniques.

Method of analysis	% Bound Mean ± S.D.
Paper - MEK	99.9 ± 0.1
Paper - saline	96.4 ± 0.3
TCA protein precipitation	87.0 ± 2.9
Sephadex G-25 gel column chromatography.	90.4 ± 1.1

A slight variation of the % ^{99m}Tc-HSA was observed as shown in Table 1 . This is in good agreement with Rhodes et al.[10] findings that ^{99m}Tc-HSA prepared by the same method gave variable results from day to day, even when prepared from the same lot of HSA kits. This variation may be due to the formation of significant amounts of insoluble technetium and unbound pertechnetate. In addition, it was found that the % ^{99m}Tc-HSA determined by TCA protein precipitation method is 10-13% less than that determined by radiochro-

matographic method. This is due to the ability of MEK to separate the $^{99m}TcO_4^-$ radiochemical impurity leaving the ^{99m}Tc-HSA, ^{99m}Tc-Sn-colloid and other reduced technetium species at the origin. With TCA protein precipitation method, only ^{99m}Tc-HSA is precipitated leaving the other radiochemical impurities such as ^{99m}Tc-Sn-colloid, free $^{99m}TcO_4^-$ and ^{99m}Tc-Sn-citrate in the supernatant.

Effect of Reaction Time :

The percent of ^{99m}Tc-HSA and the percent of different ^{99m}Tc species present in the reaction mixture were followed by paper chromatography-MEK system as a function of time . Table 2 shows the presence of ^{99m}Tc-citrate which moves with R_f 0.25-0.33 in agreement with the published value [18]. The ^{99m}Tc-HSA which stays at the origin with R_f 0.0 and the free $^{99m}TcO_4^-$ moves with the solvent front with R_f 1.0.

Table 2 : Effect of reaction time on the % ^{99m}Tc-HSA.

React time, min.,	% ^{99m}Tc-HSA, R_f 0.0	% ^{99m}Tc-citrate R_f 0.25-0.33	% $^{99m}TcO_4^-$ R_f 1.0
5	62.0 ± 0.8	36.9 ± 0.8	1.4 ± 0.5
10	95.3 ± 0.8	2.6 ± 0.8	0.5 ± 0.1
30	98.8 ± 0.4	---	1.4 ± 0.5
60	98.1 ± 0.1	---	1.9 ± 0.8

It is clear from these data that the labelling of HSA with ^{99m}Tc using Sn(II) citrate proceeds via the formation of

^{99m}Tc-citrate intermediate complex. The % ^{99m}Tc-citrate complex is decreased while ^{99m}Tc-HSA percentage is increased with time. Although 5 min is enough for reduction of TcO_4^-, 30 min is still required for the complete formation of ^{99m}Tc-HSA complex.

Effect of Sn(II) Citrate Content :

The effect of increasing Sn(II) citrate content on the % labelling of ^{99m}Tc-HSA is studied in order to determine the optimum quantity of Sn(II) citrate which gives high labelling yield. Trisodium citrate is used to dissolve and stabilize the Sn(II) citrate, 20 ml of 2 % trisodium citrate is the minimum amount required for the dissolution of 100 mg Sn(II) citrate . In all cases, the pH of the reaction mixture was adjusted to 7.4 except when the effect of pH is the factor being studied.

The data show that increasing the amount of Sn(II) citrate up to 0.24 mg Sn(II) citrate will increase the % ^{99m}Tc-HSA. Increasing the amount of Sn(II) citrate to more than 0.24 mg did not show any further increase in the labelling.

Effect of HSA Content :

The data indicate that the % labelling of ^{99m}Tc-HSA is independent of the HSA concentration in the range of 20 to 100 mg in agreement with the results of De Ligny et al. [19]. Very little change of the % labelling was observed at 200 mg or at 10 mg of HSA.

Effect of pH :

The pH dependence of the % yield of ^{99m}Tc-HSA is shown in Table 3 . The % labelling of ^{99m}Tc-HSA is increased up to pH 5.2. With further increase of the pH ,

there is no significant change of the % ^{99m}Tc-HSA. The data indicate that the optimum pH value for HSA labelling with ^{99m}Tc using Sn(II) citrate is 7.4 in good agreement with similar published data [15]. This is consistant with the observation that the exchange reaction with ^{99m}Tc-citrate is highly efficient at pH 7 [14]. Transfer of ^{99m}Tc from ^{99m}Tc-citrate to HSA occurred in high yield at pH range from 5.2 to 7.47.

Table 3 : Effect of pH on the % labelling of ^{99m}Tc-HSA using Sn(II) citrate.

pH	% ^{99m}Tc-HSA
1.40	58.2 ± 1.4
2.37	79.9 ± 1.3
5.20	99.3 ± 1.2
7.09	98.5 ± 0.4
7.47	99.9 ± 0.1
8.95	97.9 ± 0.3

Biodistribution Study :

Biodistribution at 30 min was determined in normal mice. A significantly high concentration of the injected ^{99m}Tc-HSA was found in the blood. The percentage of the injected ^{99m}Tc-HSA in the liver did not exceed 10 % indicating that no insoluble ^{99m}Tc species were present [10]. The percentage of the localized activity in the bone was taken as a measure of the activity localized in the bone marrow. Less than 2% of the injected ^{99m}Tc-HSA was observed. This is a good indication that the labelled albumin is of good quality.

It is clear from the data that all the obtained values are within normal limits [10] except the activity in the GIT which shows somewhat high value.

Stability of the Labelled ^{99m}Tc-HSA :

The labelled ^{99m}Tc-HSA prepared by using Sn(II) citrate method was found to be stable at neutral pH for 6 hrs after the addition of $^{99m}TcO_4^-$ which is suitable for the clinical application. Beyond 6 hrs, an increasing amount of the reduced hydrolyzed ^{99m}Tc complex species is observed. No increase of free $^{99m}TcO_4^-$ is observed.

Effect of Ascorbic Acid :

It was found that ascorbic acid in the concentration studied has no effect on the labelling efficiency of ^{99m}Tc-HSA prepared by Sn(II) citrate method but it has adverse effect on its biological distribution as shown in Table 4 . This is evident from the decrease in the blood activity and the increase of urine activity.

The results presented in Table 4 indicate that ^{99m}Tc-ascorbic acid complex may be formed although it is not detected by the chromatographic method used.

Summary :

The results of this study show that HSA can be successfully labelled with ^{99m}Tc using Sn(II) citrate at neutral pH. The optimum conditions for labelling were 20 mg HSA,0.24 mg Sn(II) citrate, 1.6 mg trisodium citrate dihydrate and pH 7.4. 30 min reaction time was found sufficient for the complete formation of ^{99m}Tc-HSA complex. The problem of denaturation of albumin has been resolved by labelling it at neut-

Table 4 : Biological distribution of ^{99m}Tc-HSA prepared by Sn(II) citrate with and without 0.5 mg ascorbic acid.

Organ	% Injected dose per organ without ascorbic acid	% Injected dose per organ with ascorbic acid
Liver	8.6 ± 0.3	10.4 ± 0.4
Spleen	0.8 ± 0.3	0.9 ± 0.1
Lungs	1.8 ± 0.1	1.2 ± 0.2
Kidneys	6.6 ± 0.1	7.1 ± 0.5
GIT	6.3 ± 1.2	8.7 ± 0.9
Blood	30.3 ± 0.2	21.9 ± 4.0
Heart	0.9 ± 0.9	0.5 ± 0.1
Carcass	31.3 ± 1.5	28.3 ± 0.9
Urine	13.6 ± 0.3	21.7 ± 0.9

ral pH. The labelled product was stable for 6 hrs after the addition of $^{99m}TcO_4^-$. The labelled albumin is of good quality as shown from its biological distribution.

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