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Original Article

Labeling of Human Serum Albumin with Stable Isotope of Bromine; an in Vitro Study

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Abstract

Background: Possibility to trace-label albumin with isotopes results in information concerning its synthesis, breakdown, and distribution in the intra and extra cellular spaces. The iodination of albumin is a widespread procedure used in scientific studies. Bromine not only is more reactive and less expensive than iodine, but bonds more easily with many elements. Therefore, it could be a suitable tracer in labeling procedures. The present study was designed to represent a method for labeling human serum albumin (HSA) with stable isotope of bromine.

Methods: In the present study, the labeling of HSA by use of stable isotope of bromine (^{79}Br) has been sought through a series of preliminary experiments including iodination of bovine serum albumin (BSA) and iodination and bromination of BSA. The experiments were basically designed according to that of Mc Conahey and Dixon in 1966. All measurements have been obtained by inductive coupled plasma mass spectrometry (ICP/MS). Twenty protein solutions, each having 50 mg HSA dissolved in 10 ml of 0.05 M buffer (pH 7.0) were prepared. A series of calculated amounts of pure bromine was added directly to each sample. Each sample was placed in a crystallizing dish containing crushed ice to keep the reactants cold. After dialysis and final preparation of the samples, the intensities of bromine in the samples were measured.

Results: Data indicated the maximum presence of bromine in HSA samples in a ratio of 40 atoms of bromine to each mole of HSA. After dialysis, sample analysis showed that on average, about 65% of the bromine was really bound to the HSA molecules. This finding indicates that about 26 atoms of bromine were bound to each HSA molecule. Data analyzed by simple linear regression method. Results showed that each µg increase in dose leads to 0.002 unit increase in the mean of mole ratio of pure bromine (Br_2)/HSA (P=0.001).

Conclusion: The present study has unique specifications in that almost all of the labeling procedures of plasma proteins have used other elements rather than bromine and mostly radioactive isotopes instead of stable isotopes. The present paper showed a method for about minimum as well as maximum bromination of HSA (0.05 atoms- 26 atoms), within certain limits of experimental conditions. By this method, one can exactly determine how much bromine should be used to obtain a certain desired mole ratio of Br_2/HSA with no, or at least minimal, alteration of protein behavior.

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Introduction

Among different proteins, plasma proteins as the major extracellular components of the circulatory system comprise a dynamic system with various functions.[1] Serum albumin presents in the largest amount in plasma. This protein was first analyzed by Liebig and Mulder in the 1830's and developed in 1940.[2]

Albumin has been used as a general protein model in a great number of studies such as amphoteric properties, denaturation processes, conformational changes and ion-binding properties.[3-5]

Determination of serum albumin levels has been used as a practical marker of general health status in the elderly and to determine the severity of underlying diseases. Associations between dental diseases and the general health conditions have been reported. Serum albumin concentration can be used as a marker for evaluation of the relationship between the general health conditions and root caries. Changes in serum albumin concentrations are significantly associated with changes in the number of root caries lesions. So patients with hypoalbuminemia are at high risk for root caries.[6] Moreover, severe periodontitis has been associated with low serum albumin.[7] Therefore, labeling serum albumin with trace elements to measure intravascular volume of albumin could be of significance for evaluating dental root caries and periodontitis that are two common dental diseases.

The use of isotopes, particularly the radioactive ones, for labeling proteins and related compounds is now being widely adopted for many investigations in biochemistry and medicine.[8-13]

Stable isotopes as natural constituents of chemical elements, can similarly be used for biological labeling of proteins. Application of stable isotopes as tracers in trace element research dates back to 1963 when Lowman and Krivit gave the first report of stable isotope application as a tracer to the study of mineral metabolism.[14] Methodologically, recently, Inductive Coupled Plasma Mass Spectrometry (ICP/MS) is used as a general method for isotopic measurements.

The procedure of labeling serum albumin has been essentially carried out under the same general principles as those of other proteins. The iodination of human serum albumin (HSA), as a widespread procedure, has been first studied by Li.[15]

Bromine is a dense, mobile, reddish – brown liquid that evaporates easily at standard room temperature and pressure to give a red vapor. Bromine is a halogen and is less reactive than chlorine and more reactive than iodine. It bonds easily with many elements. Organic bromides are preferable to more expensive iodide containing reagents. Organobromine is used in industry, dyes, agrichemicals, pharmaceuticals, water purifications, disinfectants and medicine.[16]

Compared with iodine that is one of the most common agents for labeling, bromine seems to have advantages in labeling processes. Being more reactive and less expensive than iodine, which binds easily to many elements, made us consider that labeling HSA with a stable bromine isotope may provide wider and safer range of minimal to maximal bromine atom attachment to HSA molecules.

Therefore, in the present investigation the labeling of HSA by stable isotope of Bromine (^{79}Br) has been demonstrated for the first time.

Materials and Methods

In the present study, a method of bromination of HSA with stable isotope of bromine has been developed. Due to the uniqueness of the procedure in its own way, in order to seek a proper method of bromination, it seemed necessary to start experiments with labeling albumin with iodine that not only is a well-known and routinely used element for this purpose, but also has the advantage of having comparable behavior with bromine. The same goes for bovine serum albumin (BSA) and HSA. Being so, the experiment was designed according to two sets of preliminary standard experiments as follows:

1. Iodination of BSA by potassium iodide (KI) using the chloramine –T procedure:

This experiment is basically designed according to that of Mc Conahey and Dixon in 1966.[17] So, in each of four 15 ml Teflon beaker, 4.8-5.1 mg of BSA dissolved in 4 ml 0.05M phosphate buffer (pH 7) were placed. Each beaker was placed in a crystallizing dish containing crushed ice to keep the reactants cold. These crystallizing dishes were then placed on magnetic stirrers and 0.5 ml of 0.1 M KI solution (6.3 mg iodide) was added. During stirring, 10 µg of chloramine-T was added drop wise to the first two beakers and 1000 µg to the remaining two beakers following addition of an equal amount of sodium metabisulfite to neutralize the remaining oxidizing agent and stop the reaction after a period of two minutes to the first two beakers and five minutes to the last two. The mixtures were placed in dialysis bags for dialysis and then the bags were placed

in distilled water with several changes for a period of 24 hours to remove non-protein bound iodine. Then after confirmation of acceptable level of intensity and concentration of sodium in the samples, concentration of iodide bound to BSA was measured by ICP/MS.

2. Iodination and bromination of BSA by elementary iodine and bromine:

The design of this experiment was basically the same as that performed with previous step. This step was an evaluation phase to see if pure iodine and pure bromine can be uptaken directly by BSA. After performing these two preliminary steps, bromination of HSA by stable isotope of bromine (^{79}Br) was the last principal and final stage of the experiment. Here again twenty protein solutions of each having calculated amount of 50 mg HSA dissolved in 10 ml of 0.05 M buffer (pH 7), were prepared. A calculated amount of 80.6, 105.4, 527, and 6345 µg of pure bromine was added directly to each set of samples. The conditions of the experiment were the same as those of previous steps. After dialysis and final preparation of the samples, the intensities of bromine were measured by ICP/MS like before. For a comparison reason and testing proportionality of different mole ratios of Br_2/HSA with respect to different concentrations of pure bromine, other series of experiments with labeling of HSA with bromide and pure bromine under different concentrations were also performed. After confirmation of the presence of bromine in HSA samples dialysis test of attachment of bromine to the HSA was performed by treating the samples with 6 M urea solution. Urea is a denaturing agent and uncoils the protein. So if any free bromine is present, it will be washed out by dialysis.

Results

Data obtained from iodination of BSA by KI using the chloramine-T, showed adding a calculated amount of 6.3 mg of iodine with a mole ratio of I/BSA of 670 to BSA before dialysis, resulted in attachment of 3.02-98.29 µg of iodine with a mole ratio of about 0.3-10 to BSA after dialysis (table 1). This table shows mean of the mole ratio of I/BSA and its standard deviation to be 4.39±3.92.

Amounts obtained from iodination and bromination of BSA using iodine and bromine, showed adding 45 µg of iodine with a mole ratio of pure Iodine (I_2)/BSA of 4.52 to BSA before dialysis resulted in attachment of 38.1-43.8 µg of iodine with a mole ratio of 3.81-4.51 to BSA after dialysis (table 2). This table shows mean of the mole ratio of I_2/BSA and its standard deviation to be 4.10±0.36.

Accordingly, 563.3 µg of bromine with a mole ratio of pure bromine (Br_2)/BSA of 46 before dialysis resulted in attachment of 93.6-135 µg of bromine with a mole ratio of 15.4-22.5 to BSA after dialysis (table 3). This table shows mean of the mole ratio of Br_2/BSA and its standard deviation to be 18.90±3.55.

Data obtained from bromination of HSA by bromine showed adding a minimal calculated

Table 1: Amounts of iodine and mole ratios of I/BSA before and after dialysis.

BSA Samples	Amount of I before dialysis (µg)	Amount of I After dialysis (µg)	Mole ratio I/BSA before dialysis	Mole ratio I/BSA after dialysis
#1	6300	3.02	670	0.324
#2	6300	68	670	7.23
#3	6300	56	670	5.96
#4	6300	9.80	670	1.04
#5	6300	3.56	670	0.378
#6	6300	98.29	670	10.45
#7	6300	50.36	670	5.36

I/BSA : Iodine/bovine serum albumin, Mean of the mole ratio of I/BSA = 4.39, Standard deviation = ±3.92

Table 2: Amounts and mole ratios of I_2/BSA before and after dialysis.

BSA Samples	Amount of I_2 before dialysis (µg)	Amount of I_2 After dialysis (µg)	Mole ratio I_2/BSA before dialysis	Mole ratio I_2/BSA after dialysis
#1	45	43.8	4.52	4.51
#2	45	38.1	4.52	3.81
#3	45	39.7	4.52	3.98

I_2/BSA : Iodine 2/bovine serum albumin, Mean of the mole ratio of I_2/BSA = 4.10, Standard deviation = ±0.36

Table 3: Amounts and mole ratios of Br_2/BSA before and after dialysis.

BSA Samples	Amount of Br_2 before dialysis (µg)	Amount of Br_2 After dialysis (µg)	Mole ratio Br_2/BSA before dialysis	Mole ratio Br_2/BSA after dialysis
#1	563.3	117.4	46	18.8
#2	563.3	93.6	46	15.4
#3	563.3	135	46	22.5

Br_2/BSA: Pure bromine **/** bovine serum albumin, Mean of the mole ratio of Br_2/BSA = 18.90, Standard deviation = ±3.55

amount of 80.6 µg of bromine with a mole ratio of Br_2/HSA of 0.67 to HSA before dialysis, resulted in attachment of 4.62-5.64 µg of bromine with a mole ratio of 0.07-0.09 to HSA after dialysis. Also, a maximal calculated amount of 6345 µg bromine with Br_2/HSA mole ratio of 529 to HSA before dialysis resulted in attachment of 111.5-240.7 µg of bromine with Br2/HSA mole ratio of 19-40 to HSA after dialysis. Data also showed that samples treated with urea demonstrated 0.05 to 26 atoms of bromine that were really attached to each HSA molecule (table 4). In this stage, to evaluate the effect of dose (as independent variable) on mole ratio (as dependent variable), we analyzed the data according to simple linear regression method. Results obtained via this linear regression method showed that for each µg increase in dose, mean mole ratio of Br_2/HSA would be increased by 0.002 unit which was in accordance with the objective of this study (P=0.001)

Discussion

The significance of labeling of proteins (including plasma proteins and in particular that of human serum albumin) with different radio isotopes (mostly those of iodine) is well accepted by all investigators.[9,11-13,15,17] The literature reflects an increasing interest in this field. This interest stems from valuable information obtained from the application of these isotopes in different fields of science. Cohen reported that radioactive iodine probably constitutes the most available and convenient label for plasma proteins.[18]

Bocci showed high labeling efficiency (60-65%) of serum proteins labeled with 131I by using the chloramine-T method.[19] Crandall et al. evaluated the effect of radio iodination and fluorescent labeling on albumin.[20] McFarlane showed that with only 5-10 atoms of iodine bound, iodinated albumin became antigenic.[21]

Sterling, Berson et al. and Munro showed the metabolism of albumin would not be altered by labeling of not more than 2-3 iodine atom per molecule.[22-24] Generally speaking, in labeling of proteins with radioactive iodine, which has widespread use in the literature, the common value of attachment of iodine atoms to each protein molecule is around 0.5-2 with the maximal permissible ratio being about 5 atoms.[25] The procedures designed for labeling of proteins even nowadays are basically the same as those stated in the above mentioned references.

Results of the first stage of this experiment, which is basically based upon radio iodinated labeling method, showed the iodine was attached to the protein and an increase in the

Table 4: Amounts and mole ratios of Br_2/HSA before and after dialysis for brominated HSA of different bromine concentrations.

Samples	Amount of Br_2 before dialysis (µg)	Amount of Br_2 After dialysis (µg)	Mole ratio Br_2/HSA before dialysis	Mole ratio Br_2/HSA after dialysis	Mole ratio Br_2/HSA after dialysis urea-treated samples
HSA + 80.6 µg bromine					
#1	80.6	5.17	0.67	0.08	0.05
#2	80.6	5.64	0.67	0.09	0.75
#3	80.6	4.42	0.67	0.07	0.04
#4	80.6	4.62	0.67	0.08	0.05
#5	80.6	5.00	0.67	0.08	0.05
HSA + 105.4 µg bromine					
#1	105.4	30.7	8.9	5.2	3.43
#2	105.4	34	8.9	5.7	3.76
#3	105.4	37.6	8.9	6.3	4.15
#4	105.4	39.9	8.9	6.6	4.35
#5	105.4	36.6	8.9	6.1	4.02
HSA + 527 µg bromine					
#1	527	170.8	44.5	29	19.14
#2	527	180.7	44.5	30	19.80
#3	527	173.4	44.5	29	19.14
#4	527	156.1	44.5	26	17.16
#5	527	200.0	44.5	33	21.78
HSA + 6345 µg bromine					
#1	6345	213.6	529	36	23.76
#2	6345	196.9	529	33	21.78
#3	6345	111.5	529	19	12.54
#4	6345	240.7	529	40	26.40
#5	6345	230.2	529	38	25.08

Br_2/HSA: Pure bromine / human serum albumin

mole ratio was in accordance with the increase in chloramine-T concentration. Needless to mention that, existence of a linear plot of intensity of emission of sodium versus its concentration, led to the measurement of iodine concentration in the samples. Data of this stage were in accordance with those obtained by McFarlane, Sterling, Berson, and Munro.[21-24] Our iodine atom uptake per molecule of BSA was slightly higher than others, probably due to the use of ICP/MS that measures precisely the stable isotope ratios for a wider range of chemical elements.

Results of the second stage of the experiment also indicated the presence of iodine and bromine in the samples after dialysis without using an oxidizing agent. Kragh- Hansen reported that serum albumin was able to bind with high- affinity ligands.[26] Results of this stage were the same with other investigators as far as iodine uptake was considered. However, uptake of bromine was significantly increased under the same circumstances. This uptake increase was expected to happen according to bromine characteristics mentioned before.

Application of stable isotopes has some advantages over radioisotopes. The most important one is that radioisotopes have inappropriate half-lives.[27-29]

By the chronological consideration of all the experiments and the goal of the present study, and regarding the successful results obtained from the bromination of HSA by stable isotope of bromine it seemed there was no need for further experiments in this regard because through all of these experiments, it had been shown that pure bromine could be added directly to HSA giving brominated HSA solutions. But, by this method, labeling of HSA with bromine has been done to an extent which is safe regarding alteration in the protein molecule.

This method has certain and in a way, unique advantages including:

1) Easy determination of any number of the bromine attached to protein molecule, within certain limits of the experimental conditions.
2) No need for radioactive or any other extra agent in comparison with other investigations.
3) Application of the preparations to living organisms (and in particular, human beings) without the hazards of radioactivity.
4) It is simple, fast, efficient, and relatively low cost.

This study can be considered unique because almost no evidence of similar study has been found in the literature. This uniqueness makes its comparison with similar investigations difficult due to the lack of exactly comparable studies. So, initiation of the study was basically designed upon almost similar procedures,[30,31] proceeding toward its uniqueness by completion of each phase of the study. This method showed a minimum number of 0.07 atom to a maximum number of 26 atoms of bromine attachment per HSA molecule. This 0.07-26 atom attachment as the final result of the whole project shows a good index in comparison to the results obtained by others who worked with radioactive iodine labeling processes of proteins. Regarding the lack of radioactivity in this study, the maximal attachment of bromine to HSA was shown to be 26 atoms (in comparison with the maximal attachment of five iodine atoms) with no, or at least minimal, alteration in albumin behavior. To test the alteration of albumin behavior, protein denaturation of the samples were evaluated. Protein denaturation results from oxidation of labile residues and can manifest itself as an altered electrophoretic mobility, the loss of biological activity and variation in the rate of clearance of injected proteins from the blood stream. Chloramine-T technique has relatively low protein denaturation effect. In the present study, after uptake of bromine, no turbid or cloudy solutions and no deposits were seen in the samples indicating that an optimum concentration of bromine has been applied. Also, the behavior of labeled albumin with that of native albumin was compared in vitro by electrophoresis. Namely, electrophoresis and micro immuno electrophoresis on agar were adopted to check possible denaturation occurring in the protein samples during the bromination process. Fortunately, no significant change was seen between the behavior of labeled albumin with that of native albumin within certain limits of experimental conditions in the present study. This result was in accordance with those of other researchers testing any possible alteration in HSA molecules by using iodine as tracer element in labeling procedures.

To preserve native protein structure and conformation, proteins were analyzed by nondenaturing electrophoresis. Native protein electrophoresis was carried out in Tris-glycine electrophoresis buffer.

The present study has the advantage that one can exactly determine how much of the bromine should be used to obtain a certain desired mole ratio of Br_2/HSA after dialysis.

Needless to mention, following the preparation of labeled proteins based on the proposed model of this study, by further laboratory and animal experiments, one is able to find the optimal mole ratio of Br_2/HSA that can

be safely used.

It would be clear that upon successful demonstration of the optimal mole ratio of Br_2/HSA, prepared samples can be used in a vast number of research processes.

Conclusion

The present study showed that labeling of HSA with stable isotope of bromine resulted in a wider range of minimal to maximal bromine atom attachment to HSA molecules with no, or at least minimal, alteration of protein in comparison with the use of other similar chemical agents. Based on the results of this study, the best range of mole ratio of Br_2/HSA can be estimated. The promising results of this labeling method, make it possible in the near future to compare in vitro and in vivo behavior of these labeled protein samples with that of native proteins and upon successful accomplishment of this step, to use the labeled samples in human subjects to measure intravascular volume of albumin that can be considered a great success.

Conflict of Interest: None declared

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