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PECULIARITIES OF THE INTRODUCTION OF TECHNETIUM ISOTOPES INTO PROTEIN MOLECULES - OF HUMAN SERUM ALBUMIN AS AN EXAMPLE

by

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A B S T R A C T

The peculiarities of the introduction of the radioisotope ^{99m}Tc into the molecules of human serum albumin are being investigated. It turned out that tin not only participates in the Tc (VII) reduction process, but even is being introduced into the originating Tc-albumin complex. It is shown that no more than four technetium atoms enter into bond with an albumin molecule. The authors express their opinion that in order to produce high quality protein preparations, the albumin has to be modified through a polyfunctional complexing agent, which forms an entirely saturated coordination complex with Tc(IV).

Determination of peculiarities of introduction of isotopes of technetium into the molecules of organic and natural compounds, in particular in albumin, presents in the first place an important scientific problem. However, the majority of work published in the last years are dedicated mainly towards solving of applied problem - obtaining of concrete albuminous compounds labelled by the isotope of technetium 99m Tc. The first step in various methods of introducing isotope of 99m Tc into the molecules of albumin lies in primary reduction of TcO_4^- eluting as solution of pertechnetate-anion from the column of the standard generator, to the lower valency states the most probable of which is Tc (IV) and following, combining it with various chelates. It is known that Tc (IV) due to the structure of its outer shell of electrons, is able to form complexes with chelates of various kinds (1, 2). This property of Tc (IV) is widely utilised in radiopharmaceutical practice for obtaining various compounds labelled with short lived isotope of 99m Tc. Nevertheless, essentially there is no single radiopharmaceutical compound labelled with 99m Tc in the starting point of which there would be given clear concept (plan) on obtaining stable bond between Tc and chelates and distinguishing itself by a strong radioactive label.

Reduction of Tc(IV) is conducted (carried out) with various metals of variable valency, particularly with cations of Sn(II). Described is a number of methods by which reduction of Tc(VII) is accomplished by chemical reactions of freshly prepared chloride of Tin $SnCl_2$ with the solution of pertechnetate (3). Besides that reduction of Tc(VII) by cations of Sn(II) or of other metals (Fe, Zr), forming in solution with (bY) electrochemical dissolving (diffusion) metalic anodes was investigated (4-7)..

It is known that in the medium (atmosphere) of hydrochloric acid (pH 1-1,3) electrolytic dissolving of Sn(II) is not accompanied by hydrolysis (8). In some cases to prevent hydrolysis of Sn(II) metallic tin is added to the solution (8).

During investigation of conditions of introduction of 99m Tc into the molecules of protein in albumin the role of tin or other metals of variable valency in bonding Tc(IV) with proteins was examined (5). Proposals were made that joining of Tc(IV) proceeds with carboxyl, disulfide, sulfide, and other functional groups of molecule of albumin (4).

In the present work on example of blood albumin serum, we attempted to study a number of questions, which in our view, are fundamental in understanding the bond between albumin and Tc.

(a) What is the minimum quantity of tin required for full reduction of TcO_4^-;

(b) role of Sn(II) in inclusion of 99m Tc into molecule of albumin namely, does the Sn(II) take part in formation of complex with Tc or does its role lead only to the simple reduction of TcO_4^-.

(c) limiting number of atoms of Tc which can be included in molecule of albumin in the presence of Sn;

(d) what functional grouping in albumin molecule takes part in the process of bonding Tc(IV);

(e) Strength of bond Tc(IV) with various chelates of albumin molecule.

Question of quantity of tin necessary for the full reduction of TcO_4^- in scientific literature is not considered in detail. Usually for the reduction a large surplus of $SnCl_2$ is utilised. According to authors (9) in the atmosphere of hydrochloric acid (pH_3) reduction of Tc(VII) flows to Tc(IV). Hence we have studied this process assuming validity of stoichiometric relationship (1).

$$3\ Sn(II) + 2\ Tc(VII) \rightarrow 3\ Sn(IV) + 2\ Tc(IV) \qquad (I)$$

Pertechnetate of potassium (99m Tc) and pertechnetate of sodium (99m Tc) were reduced at pH 1-1.3 with electrically generated cations of Sn(II). Microsamples for estimate of relationship between Tc(VII) and Tc(IV) at various time intervals were taken from the cell for analysis. Results of this investigation are given in Table 1.

It may be seen from Table 1 that the obtained results satisfactorily support the relationship (1) and that for reduction of two atoms of Tc(VII) three atoms of Sn(II) are necessary. This result for each concrete case should be utilised with certain estimated corrections.

Caution required in the choice of the minimum magnitude of current flowing through the cell is due to the fact that impurities present in elute of the generator of 99 Mo 99m Tc have influence on its magnitude. Authors (4) recommend repeated washing with isotonic solution to eliminate traces of acidifiers, however, this is not always effective. Besides this to simplify cell construction electrolytic dissolving of tin is carried out without separating electrodes by diaphragm, and as a result part of generated on anode cations of Sn(II) is reduced on cathode and generally content in the solution may not be proportional to the passing quantity of current. Presence of surplus of cations Sn(II) in acid solution in binding of Tc(IV) with proteins in albumin may lead to lowering functional suitability of the obtained compound. This is connected with the fact that Sn(II) hydrolises with neutralisation of the solution and forms non organic colloidal solutions (compounds) which can aggregate (unite) with protein in albumin molecule.

Study of only some factors which influence conditions of obtaining electrolitically Sn(II) shows necessity of experimental determination of the magnitude of electric current in given conditions.

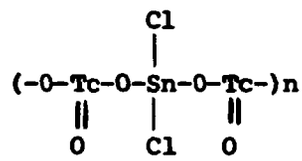
To clarify the role of tin in the process of binding Tc with albumin we have studied the possibility of producing (generating) of complex between Sn and albumin indirectly. Cations of Sn(II) were mixed with solution of albumin and were kept for 10-15 minutes, the mixture was subjected to gelfiltration and content of tin in albumin fraction was determined. Obtained results are given in Table 2.

From the results in Table 2 it is obvious that in these conditions only an insignificant number of atoms of tin bind to albumin (average one atom of tin to 10-15 molecules of albumin) and obviously do not form a chemically bonded complex.

If pertechnetate of potassium (99 Tc) is reduced by cations of Sn(II) and then is mixed with the solution of albumin, then the content of tin in the fraction of albumin increases and constitutes an average 1 atom of tin to two molecules of albumin, containing stable (secure) 99 Tc (Table 3).

Such diversity in degree of including tin in molecule of albumin and complex - Tc points to the fact that tin does not form a strong chemical bond with molecule of albumin, but takes part in the process of reduction of TcO_4^- and is included (is incorporated) into forming complex of albumin with technetium.

Reduction of Tc(VII) by cations of Sn(II) is accompanied by evolving (generation) divalent and trivalent and other compounds (amalgamation) containing Sn and binding with molecules of albumin. Simpler form of such binding is possible to represent by structure :-



where number n representing length of chain depends on conditions of conducted reaction and in the first place on the pH value of the solution. Neutralisation of surplus acidity in the solution leads to hydrolysis of binding Sn-Cl, so in $SnCl_2$ as in divalent and trivalent compounds Tc with tin and formation of colloidal particles which substantially show up in quality of preparation (compound), colloidal particles on introduction of solution in living organisms accumulate in liver.

We have also made an attempt to determine the maximum number of atoms of Tc which could be included in one molecule of albumin. For this we have utilised weight property of $KTcO_4$ (99 Tc) and for determination of its concentration we have added solution of 99m Tc. Various molar relationships of 99 Tc and albumin in parent (original) solution were also investigated. Results of these experiments are given in Table 4.

These results show that no more than four atoms of technetium can be bound to one molecule of albumin.

It is known that a number of aminoacids entering into the structure of albumin have "bidentatic" groups capable of forming two or more coordinated compounds with complexing metal. For comparison of their relative abilities to form coordinated compounds with technetium reduction of solution of Tc(VII) to Tc(IV) was carried out with following addition of aminoacids. In experiments carrier free 99m Tc in the presence of surplus of aminoacids was utilised. Magnitude of activity included (contained) in complexes of aminoacids with 99m Tc being formed was determined using autoradiography. As a result of such investigation it was found that valine, methionine, serine and histidine do not form strong chemical complexes with Tc(IV). In practice full binding of Tc(IV) in analogous condition was observed only with cysteine. This

indirectly points to the preferential role of sulfhydryl groups in the process of binding isotope of technetium with albumin.

As is known from literature (10) one molecule of albumin contains four cysteine aminoacidic groups. For obtaining a fully saturated coordinated complex with technetium presence of at least two neighbouring groupings such as -SH $-NH_2$ and others, is essential. Taking into account sequentiality of aminoacids in polypeptide chain it may be expected that four atoms of technetium which are included in molecule of protein of albumin will coordinate with -SH group and other neighbouring functional groups of the protein in albumin molecule in various combinations. This circumstance permits to suggest that strength of binding of atoms of technetium with various parts of protein in albumin molecule will also vary.

Feasibility of exitence of this way bound technetium to protein including albumin in living organism is not very likely because atom of Tc may either interact with other chelates yielding (giving) coordinated saturated complexes or splits because of oxidation by oxygen present in blood channel. Confirmation of this is observed by us picture of behaviour of various samples of albumin labelled with 99m Tc in living organisms (table 5).

As may be seen from table 5 content of Tc in blood channel decreases with time, by about factor of two in 60 minutes, and elimination of it with urine and an increase of activity in liver is observed.

We think (suppose) that :-

1. By way of interaction of reduced Tc(IV) with protein in albumin molecule it is impossible to obtain sufficiently strong stable label of 99m Tc for compounds utilised for diagnostic purposes for intraveneous introduction.

2. One way of creating high quality protein compound (preparation) in our view, appears modification of albumin by polyfunctional complexing agent which may form fully saturated strong coordinated complexes with Tc(IV) and with other elements.

EXPERIMENTAL PART

In investigation we utilised solution of carrier free pertechnetate of sodium (99m Tc), elurating from generator (99 Mo); pertechnetate of potassium (99 Tc); solution of albumin of human blood serum (ACKЦ) with concentration of 200 mg/ml in water containing 0.9% NaCl.

Introduction of isotope of technetium into albumin and model aminoacids was carried out in electrolitic cell (medical flask) which contained tin electrodes (wire of 0.8 mm diameter) fixed in lid holder of the flask.

As source of direct current was utilised instrument with potential 0.5 - 1.6 volt. Magnitude of current was controlled by ammeter and was regulated by potentiometer 0.5 - 1.6 mA.

In the cell containing solution of isotope of technetium (1 - 5 ml) was added 1 n of chydrochloric acid 0.5 - 1ml to pH 1 - 1.3, electrodes holder was inserted, current was switched on and its magnitude was controlled. Solution of albumin was brought into the cell before beginning of electrolysis and/or after its termination depending on conditions (specification) of the experiment. Neutralisation of solution to pH 5 - 7 was carried out with 0.5 n solution of NaOH. During entire process the solution was stirred with magnetic stirrer. Analysis of end product interaction on content of now reduced TcO_4^- was carried out in accordance with the generally accepted method : chromatography on paper (Batuman - 1) in the medium (atmosphere) 85% strength methanoyl (3). Solution of albumin and model aminoacids labelled with the isotope of technetium was analysed by electrophoresis in borate buffer (I think it means shielded by borate) (pH 8.3) with 450V during 1 hour with the subsequent radiographing of suitable (matching) spots on chromatograms.

For separation and identification of components of solution containing albumin bound with isotope of technetium gelchromatography was utilised using acrylamidic gel P-2 (150 x 10 mm). Time of elution of albumin fractions and concentration of albumin was determined using spectrophotometers SF-4 and Gilford-2000; to control separation of these fractions radiometering attachment was used.

General content of tin in the solution of albumin labelled with 99 Tc and 99m Tc was determined by method of emissive spectral analysis on quartz spectrograph ISP-30, sensitivity of which works out at 0.25 - 0.5 mkgr/ml with reproductability of results $\pm$ 10%.

Rats were used in these investigations of specis (nonspecified) and Wister, weighing 180-200 g. Specimen of solution of albumin labelled 99m Tc were introduced internally (intraveneously) in quantity of 0.2 ml. In set intervals of time the animals were killed by decapitation and by method of direct radiometering content of technetium in investigated organs was determined. General volume of circulating blood was taken as 7% of body weight, for radiomeasurement 1 ml of blood was taken.

TABLE 1

QUANTITY OF TIN NECESSARY FOR A FULL REDUCTION OF Tc(VII)

$$3\ Sn(II) + 2\ TcO_4^- \rightarrow 2\ TcO_2 + 3\ Sn(IV)$$

CONTENT OF $KTcO_4$ 99 Tc mg	QUANTITY OF ELECTRIC CURRENT COULOMB	QUANTITY OF Sn ACCORDING TO EQUATION 1 mg	DISTRIBUTION OF ACTIVITY BETWEEN Tc(VII) & Tc(IV)%
0.4	0.29		70/30
	0.58		51/49
	0.87		29/71
	1.17	0.72	12/88
2.0	1.45		78/22
	2.90		50/50
	4.35		16/84
	5.80	3.6	9/91
10.0	7.37		77/23
	14.75		62/38
	22/12		17/83
	29.50	18.0	7/93

TABLE 2

QUANTITY OF TIN BOUND WITH ALBUMIN (ACKЦ)* (ASBH)

QUANTITY OF ELECTRICITY COULOMBS	CONCENTRATION OF (ACKЦ) IN SOLUTION mg/ml		CONCENTRATION OF Sn IN SOLUTION mg/ml		MOLAR RELATIONSHIP Sn : ACKЦ*	
	INITIAL	AFTER GEL-CHROMATOGRAM	INITIAL	AFTER GEL-CHROMATOGRAM	INITIAL	AFTER GEL-CHROMATOGRAM
0.05	10	1.74	3.08	0.25	1:5.5	1:12
0.05	10	3.10	3.08	0.30	1:5.5	1:17
0.10	10	1.96	6.16	0.25	1:2.7	1:15
0.10	10	2.23	6.16	0.25	1:2.7	1:15
0.50	10	2.18	30.8	0.25	1.8:1	1:15
2.0	10	1.47	123.2	0.25	7.2:1	1:10

*Russian abbreviation of Albumin, Serum, Blood Human of which english equivalent is Human Serum Albumin.

TABLE 3

CONTENT OF TIN IN ALBUMIN LABELLED WITH 99 Tc

CONTENT OF $KTcO_4$ (99 Tc) mg/ml	QUANTITY OF ELECTRIC CURRENT COULOMB	CONTENT OF Sn mkg/ml		CONCENTRATION ACKU mg/ml		MOLAR RELATIONSHIP Tc : ACKU	MOLAR RELATIONSHIPS Sn : ACKU - Tc	
		INITIAL	AFTER GEL-CHROMOTO-GRAM	INITIAL	AFTER GEL-CHROMOTOGRAM	INITIAL	INITIAL	AFTER GEL-CHROMOTOGRAM
0.102	1.46	76.0(90)*)	5.40	37.4	7.64	1:1	1.2:1	1:2.4
0.020	0.29	28.8(18.0)	1.66	5.15	2.33	1:0.7	3.2:1	1:2.4
0.102	1.46	46.0(90.0)	3.50	21.8	5.75	1:0.6	1.25:1	1:2.8
0.205	2.92	60.0(180)	9.80	39.0	14.9	1:0.5	0.0:1	1:2.6
0.102	1.46	31.1(90.0)	4.26	14.0	4.05	1:0.4	1.5:1	1:1.65

*Lowered content of tin in solution, experimentally determined, conditioned by various contents of other cations in original generator.

TABLE 4

LIMITING NUMBER OF Tc(IV) MOLECULE BINDING WITH ONE MOLECULE OF ALBUMIN (ACKU)

CONTENT OF $KTcO_4$ (99 Tc) mg/ml	CONCENTRATION OF ACKU IN SOLUTION mg/ml	R/ACTIVITY ACKU - Tc RELATIVE TO INITIAL ACTIVITY OF PERTECHNITATE (TcO_4) %	MOLAR RELATIONSHIPS 99 Tc : ACKU *	
			INITIAL	AFTER GEL-CHROMATOGRAM
0.1	35.0	91	1 : 1	0.91 : 1
0.2	35.0	81	2 : 1	1.62 : 1
0.2	35.0	85	2 : 1	1.69 : 1
0.2	17.5	73	4 : 1	2.39 : 1
0.2	17.5	99	4 : 1	3.94 : 1
0.2	13.2	70	6 : 1	4.20 : 1
0.2	8.8	63	8 : 1	3.96 : 1

* Isotope 99m Tc from generator was utilised as indicated in radiometric measurements.

TABLE 5

CONTENT OF 99m Tc IN SOME ORGANS OF A RAT AFTER INTRODUCTION INTERNALLY, SPECIMENS OF ALBUMIN LABELLED WITH TECHNETIUM - 99m

NO. p/p	TIME OF DECAPITATION min	ORGANS AND TISSUE			
		BLOOD	LIVER	KIDNEY	LUNGS
1.	15	87.8 ± 0.5	14.3 ± 0.4	7.8 ± 0.5	6.1 ± 0.8
	60	30.3 ± 2.7	19.4 ± 1.0	12.0 ± 0.6	3.4 ± 0.3
2.	15	68.5 ± 0.2	9.4 ± 1.1	7.2 ± 0.5	6.3 ± 0.1
	60	52.3 ± 2.2	9.0 ± 0.4	11.0 ± 1.0	2.2 ± 0.4
3.	15	78.0 ± 6.5	14.0 ± 0.7	10.0 ± 0.4	6.1 ± 0.8
	30	68.0 ± 0.7	15.0 ± 0.7	9.0 ± 0.6	4.2 ± 1.2
	60	24.0 ± 5.6	8.0 ± 1.9	5.2 ± 0.4	1.6 ± 0.4
4.	15	51.0 ± 1.4	7.0 ± 0.6	8.0 ± 0.9	5.0 ± 1.0
	30	43.7 ± 1.3	6.0 ± 0.0	7.0 ± 0.4	3.0 ± 0.6
	60	37.6 ± 2.7	6.0 ± 0.6	12.0 ± 0.8	4.0 ± 0.7
5.*)	10	37.2 ± 3.1	4.6 ± 0.3	8.6 ± 0.7	
	30	22.5 ± 1.0	3.5 ± 0.5	14.1 ± 1.1	
	60	13.5 ± 1.3	3.0 ± 0.1	14.9 ± 0.4	

*For comparison results on distribution of radioactive compound of albumin - 99m Tc by firm "cea-ire-sorin" were taken (brought in).