THE USE OF SEPHADEX IN THE CHROMATOGRAPHY OF THYROXINE-CONTAINING COMPOUNDS: A CRITIQUE

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Many papers have been published on the separation and estimation of iodide, free thyroxine and plasma-protein-bound thyroxine by means of gel filtration on Sephadex[1-5]. The aim of the present paper is to give a qualitative assessment of the methodical errors in the procedure, arising from the chemical properties of the thyroxine and the Sephadex material, which have to be taken into account in the evaluation of the results.

MATERIALS AND METHOD

Sephadex G-25, medium (AB Pharmacia, Uppsala), *d,l*-thyroxine T4 (pro injectione, 1 mg/ml, Roche, Switzerland), Tris-hydroxymethylaminomethane, TRIS (Lachema, Czechoslovakia), ^{131}I-*d,l*-thyroxine, radiothyroxine, RT4 (Radiochemical Centre, Amersham, England), and blood plasma from one subject were used. Tracer amounts utilized in single experiments did not exceed, even after RT4 degradation, the concentration of 0.1 μg of T4 or 1 μC of radioactivity.

A TRIS–HCl buffer solution of pH 7.4 and ionic strength 0.05 according to GOMORI[6] was used. Chromatographic columns measuring 1 cm × 10 cm ("small") and 2 cm × 20 cm ("large") in diameter, with a glass sintered end plate, were filled with Sephadex gel as described by FLODIN[7]. Buffer was pumped through the column by means of a micropump at a rate of 1 ml per min. A thin glass tube (diam. 2 mm) attached to the bottom of the column passes through the well of a crystal scintillation counter and then to a sink. The radioactivity of the passing fractions was registered by the impulse counter (TESLA), connected to an integrator N-30 (METRA)[8]. Various mixtures of RT4, T4 and blood plasma were applied to the top of the column in TRIS buffer and were separated on passing through the column. The radioactivity of the emerging fractions passing through the counter was registered continuously. Each mixture was incubated for at least 30 min at room temperature in order to attain an equilibrium between the free and the bound form of T4. The areas of the individual chromatographic peaks, representing the radioactivity of individual fractions, were evaluated by planimetry.

RESULTS AND DISCUSSION

When a mixture of T4, labelled by RT4, and blood plasma is incubated, a considerable amount of thyroxine becomes bound to some of the proteins of the blood

plasma (protein-bound thyroxine, PBT4). Only about 0.1 % T4 remains unbound at physiological concentrations[8]. It may be presumed that a dynamic equilibrium exists between free thyroxine (FT4), PBT4 and the corresponding binding proteins, according to the law of mass action. An equilibrium of a similar type has also been observed between the polydextran gel of Sephadex and T4. If then a sample of plasma, preincubated with T4 and labelled with RT4, is transferred to the top of the Sephadex column, PBT4 will be first to elute from the column, when the latter is washed with buffer, followed by low-molecular iodide. FT4, adsorbed on polydextran gel, will be eluted from the column by washing with blood plasma.

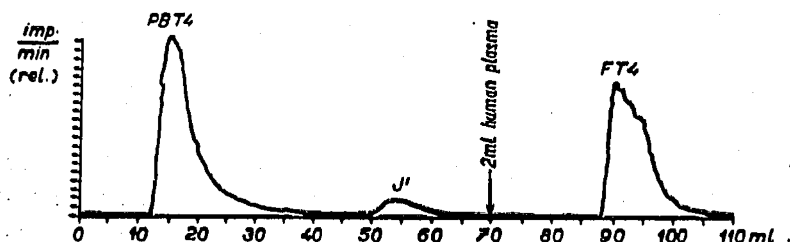

Fig. 1. Example of a separation of FT4, PBT4 and I^- in plasma on a "large" Sephadex column. Radioactivity of the fractions flowing out of the column is measured in relation to the volume. The peak on the left is formed by PBT4, the middle peak belongs to the low-molecular iodide fraction. After washing the column with 2 ml of blood plasma, unbound thyroxine is eluted, represented by the peak on the right.

Effect of the presence of radioiodine

A mixture of 15 ml of human blood plasma, 15 ml of TRIS buffer and 15 μC (0.15 ml) of RT4 was incubated. One ml of this mixture was separated on the "large" Sephadex column. The pattern of the outflowing radioactive fractions is shown in Fig. 1. In spite of the fact that a freshly obtained RT4 was used (supplied on the day of the experiment) the presence of radioactivity was evident in the spots where iodides moved. RT4 always contains about 3–10% of radioactivity in the form of iodide[9].

TABLE I

PERCENTAGE OF IODIDE PRESENT IN RADIOTHYROXINE, DETERMINED BY MEANS OF SEPARATION ON SEPHADEX GEL COLUMN

n	$FT4$ mm^2	$PBT4$ mm^2	I^- mm^2	$(PBT4 + I^- + FT4)$ mm^2	$\frac{I^- \cdot 100}{PBT4}$ %	$\frac{I^- \cdot 100}{PBT4 + I^- + FT4}$ %
1	153	190	18	361	9.47	4.98
2	152	192	18	362	9.37	4.97
3	130	237	27	394	11.39	6.85
4	120	226	23	369	10.17	6.23
5	115	205	15	335	7.31	4.47
6	136	214	27	377	12.61	7.16
				Mean ± S.D. =	10.05 ± 1.82	5.77 ± 1.11

This table and the following tables show the relative amount of individual substances; mm^2 represents the area of the peak of radioactivity registered by the counter during the flow-through of the fractions of individual substances.

Table I shows the areas of the peaks FT4, PBT4 and I^-, in mm^2, representing the radioactivity and thus also the relative amount of the individual emergent fractions.

Thyroxine itself, as used in the tests, is not pure, but contains measurable quantities of iodide, which have to be taken into account in the evaluation of the results. In relation to PBT4, approximately 10% of radioactivity comes from iodides, in relation to the total amount of added RT4, about 6% is represented by radioactivity due to iodides.

Effects of the shape and size of the column

(*a*) *Separation of iodides.* One ml of a mixture of RT4 and blood plasma in TRIS buffer was separated on each of the two Sephadex columns, *i.e.* the "large" and "small" columns as described above. While the iodide fraction in the "large" column was completely separated from PBT4 (Fig. 1), these two were liberated together in one fraction in the "small" column (Fig. 2).

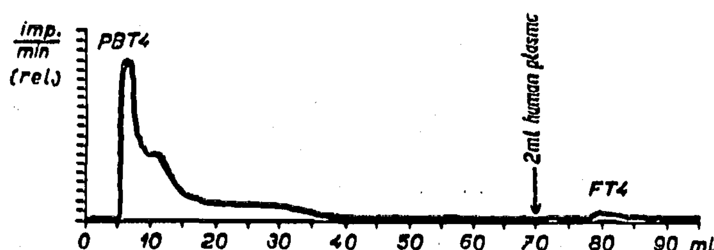

Fig. 2. Separation of a mixture as in Fig. 1 on a "small" column. On the left, double peak of PBT4 and I^-; following washing with 2 ml of blood plasma, FT4 is eluted, represented by the peak on the right.

If we wish then to evaluate the amount of unbound T4 in relation to that bound to proteins, according to the separations achieved on small columns, an error will be included in the results owing to the presence of various amounts of iodide, which apparently increase the amount of PBT4. The amount of FT4 in percentage may be calculated according to the two formulae:

$$\text{FT4}\,\% = \frac{(\text{FT4}) \cdot 100}{(\text{PBT4}) + (\text{I}^-) + (\text{FT4})} \quad (1)$$

$$\text{FT4}\,\% = \frac{(\text{FT4}) \cdot 100}{(\text{PBT4}) + (\text{FT4})} \quad (2)$$

The more exact of the two is formula (2), which eliminates the error caused by the presence of nonthyroxine iodide fraction. From this point of view it is expedient to utilize a column sufficiently large to permit a complete separation of the low-molecular iodide fraction.

(*b*) *Thyroxine adsorption on Sephadex.* As the PBT4 fraction descends through the column a readjustment of the PBT4 and FT4 ratio takes place according to the law of mass action. On a longer column, PBT4 comes more into contact with Sephadex and splits off relatively more FT4 than on a smaller one. The ratio FT4/PBT4 +

FT4, calculated from results of the separation on a smaller column is closer to the real ratio of both these substances in the original solution of the sample.

Table II shows the percentage amounts of FT4, calculated from separations on a "small" and a "large" column.

Working error of the method

Six successive separations, involving 1 ml of RT4 mixture each, were performed on the "large" column according to the conditions described above. The percentage of FT4 was determined according to formula (2). The percentage error according to column 3 in Table II is 11.4 %.

TABLE II

PERCENTAGE OF FREE THYROXINE IN PLASMA SAMPLE WITH ADDED RT4, DETERMINED ON TWO DIFFERENT COLUMNS

n	Size of the Sephadex column		
	"small"	"large"	"large"
	$\frac{FT4 \cdot 100}{PBT4 + I^- + FT4}$ %	$\frac{FT4 \cdot 100}{PBT4 + I^- + FT4}$ %	$\frac{FT4 \cdot 100}{PBT4 + FT4}$ %
1	1.90	42.38	44.60
2	10.52	41.98	44.18
3	1.77	32.99	35.42
4	4.37	32.52	34.88
5		34.32	35.93
6		36.07	38.85
Mean ± S.D.	4.64 ± 4.1	36.71 ± 4.42	38.94 ± 4.45

Effect of the quantity of T4 present in the mixture

It may happen that due to insufficient washing with blood plasma part of FT4 is not eluted from the column when samples containing large amounts of T4 are to be separated[5]. This FT4, remaining on the column, will affect the next separation by increasing the amount of FT4 in this next sample.

Two separations, each involving 1 ml of mixture A (consisting of 2.0 ml of blood plasma, 2.0 ml of TRIS buffer and 2.0 μC (0.02 ml) of RT4) were carried out on the "large" Sephadex column. Following each separation, the column was washed with 2 ml of blood plasma and the results registered. Then 1 ml of mixture B (consisting of 1.0 ml of plasma, 1.0 ml of solution T4 at a concentration of 1 mg T4/ml and 1.0 μC of RT4) was applied on the column, separated, and then washed with 2 ml of plasma. The whole procedure of separating mixture B was repeated, including the washing with 2 ml of plasma. And finally, mixture A was again separated twice in succession with corresponding washings.

The values of FT4 (calculated according to formula (2)) show that if a larger amount of T4 is present in the mixture, a single washing with 2 ml of blood plasma is not enough to clean the column sufficiently (see Table III). The FT4 fixed on the column affects the results of the following separations.

TABLE III

EFFECT OF THE PRESENCE OF THYROXINE IN THE COLUMN ON THE RESULTING VALUE OF FT4

Results of the separation of mixture A	*PBT4 mm²*	*FT4 mm²*	*FT4 %*
(1) Before the separation of mixture B	51	17	25.0
(2) Before the separation of mixture B	37	15	28.8
Mean			26.9
(1) After the separation of mixture B	33	46	58.2
(2) After the separation of mixture B	20	42	67.7
Mean			62.95

Elution of unbound T4 from the column

The T4 adsorbed on the Sephadex may be eluted by washing with buffer solution in an amount of 10–12 times the volume of the column[10]. However, it is more convenient to wash the column with blood plasma as its proteins bind T4 more firmly than Sephadex.

One ml of a solution containing 1 mg of T4 and 1 μC of RT4 was applied to the top of a "large" column. The latter was then washed with 5 $\times$ 1 ml of blood plasma. Following each washing, the column was rinsed with 20–50 ml of buffer. The radioactivity of the proteins flowing out with the bound T4 and RT4 was registered as in the preceding experiments. Table IV illustrates the separation of RT4 when the column was cleansed with blood plasma. Washing the column with 3 ml of blood plasma is adequate to clean it from 1 mg of T4.

TABLE IV

EXAMPLE OF THE ELUTION OF ADSORBED THYROXINE FROM THE COLUMN BY REPEATED WASHING WITH BLOOD PLASMA

Eluted with	*PBT4 in mm²*
1st ml of plasma	20
2nd ml of plasma	50
3rd ml of plasma	100
4th ml of plasma	0
5th ml of plasma	0

The amount of blood plasma necessary to rid the column completely of adsorbed T4 traces depends (1) upon the amount of T4 bound to the Sephadex, and (2) upon the shape and size of the column.

It may be assumed that at least a minor portion of T4 becomes bound to the Sephadex irreversibly, and we are able to elute from the column only a certain percentage of T4 adsorbed on Sephadex by means of some washing agent (albumin solution, blood plasma). This fact, however, does not interfere with the results as it is a constant; for instance, if work is being carried out with a solution of thyroxine and albumin, it will suffice to wash the column with a suitable amount of albumin solution to prepare it for further separations of albumin mixtures. The portion of T4,

irreversibly adsorbed on Sephadex, which we could elute by agents capable of binding it even more firmly, does not manifest itself at all in further tests with albumin solutions.

CONCLUSION

It is not easy to eliminate the errors of the method described here. However, relative, though valuable results will be obtained when the method is so standardized that it will yield similar results in similar experiments, within the range of deviation, even though, from an absolute point of view, these results may be accompanied by a systematic error.

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SUMMARY

A critical assessment is made of the separation of thyroxine, free and bound to plasma proteins, and of iodide by means of gel filtration on Sephadex. The following sources of error are discussed: (1) Spontaneous degradation of thyroxine to iodide. (2) The effect of the shape and size of the column on the relative proportions of the separated substances. (3) The effect of the adsorption of free thyroxine on the Sephadex material. The ways by which thyroxine is eluted from the column are also discussed and the error for the method is determined.

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