Journal of Radioanalytical Chemistry, Vol. 46 (1978) 201–209

ADSORPTION CHROMATOGRAPHIC SEPARATION OF RADIOIODINE LABELLED IODOTHYRONINES

G. TÓTH

Institute of Isotopes of the Hungarian Academy of Sciences, Budapest 1525 P.O. Box 77 (Hungary)

(Received April 28, 1978)

3,3'-diiodothyronine, 3,3',5-triiodothyronine, 3,3',5'-triiodothyronine and thyroxine was labelled with ^{125}I and/or ^{131}I by the use of the chloramine T method.[1,2] The labelled products were separated by adsorption chromatography using Sephadex LH-20 dextran gel as adsorbent and aqueous solution of ethanol as eluent.

Introduction

Dextran gels are frequently used to fractionate substances according to molecular size. In addition, dextran gels proved to be ideal as adsorbent for the separation of organic acids and bases. In the former case substances are eluted from column of dextran gel in order of decreasing molecular size, in the latter one elution order will be the sequence of the increasing adsorption affinity towards the gel.

In some cases both gel filtration and adsorption occurs on dextran gels, while in case of low molecular weight substances, which can penetrate the gel particles, separation is governed by adsorption only.

Separation of organic acids and bases is based on the finding that usually the unionised forms are firmly adsorbed on dextran gel, and ionisation results in the decrease of the adsorption affinity towards the gel and consequently the decrease of the distribution coefficient as well.[3]

Iodothyronines could thus be separated on the basis of the differing acidity of their phenolic hydroxyl group. In effect, there are two circumstances which make separation of the eight iodothyronines complicated as compared to simple organic acids.

One of them is that the eight iodothyronines form with regard of the pK value of the phenolic hydroxyl group three groups only. As it turns out from the data enlisted in Table 1, the acidity of the phenolic hydroxyl group depends on the

Table 1
Classification of iodothyronines according to the number of iodine atoms per molecule and to the number of iodine atoms in the phenolic ring.
The pK_{OH} values are from Ref.[4]

Number of iodine atoms in the outer (phenolic) ring / per molecule	0	1	2
1	3T1	3'T1	
2	3,5T2	3,3'T2	3',5'T2
3		3,3',5T3	3,3',5'T3 (rT3)
4			T4
pK_{OH}	9.3–9.5	8.3–8.5	6.4–6.7

number of iodine atoms at the 3' and 5' positions of the outer (phenolic) ring. Consequently iodothyronines enlisted in one of the three columns of Table 1, i.e. those exhibiting two, one or no iodine atoms at these positions, exhibit almost the same pK_{OH} values which render their separation based on the different adsorption affinity of the ionised and unionised forms difficult.

An other feature of the adsorption of iodothyronines on dextran gel lies in the fact that besides the pK_{OH} value the number of iodine atoms per molecule may also influence the distribution coefficient, the greater the number of iodine atoms per molecule the greater the distribution coefficient.[5–7]

The aim of this paper is to show that k values of the iodothyronines may be controlled by choosing the proper pH and ethyl alcohol concentration of the eluent.

Experimental

Nonstandard abbreviations used in this paper:
T4: L-thyroxine,
T3: L-3,3',5-triiodothyronine,
rT3: L-3,3',5'-triiodothyronine,
3,3'T2: L-3,3'-diiodothyronine,

3,5T2: L-3,5-diiodothyronine,
3',5'T2: L-3',5'-diiodothyronine,
3Tl: L-3-iodothyronine,
3'Tl: L-3'-iodothyronine.

Labelling of 3,3'T2, T3, rT3 and T4 with radioiodine

rT3 (Henning GmbH, Berlin), T3 (Sigma) and T4 (Fluka) was dissolved in a few drops of 0.1N NaOH and 5–10 μl of this solution was added to phosphate buffer (pH = 7.4). 2–3 mCi of ^{125}I or ^{131}I in 10 μl along with 25–100 μg chloramine T in 10–25 μl was introduced into the former solution, the reaction mixture was incubated for 10–50 sec and the reaction was quenched with 150–200 μg of sodium metabisulphite in 100 μl.[1,2]

Radioiodine labelled 3,3'T2 was formed as a by-product in the course of labelling rT3 with radioiodine.

Column chromatography

Sephadex LH-20 dextran gel was used as packing. The gel was allowed to swell in distilled water for 12–24 hrs and then it was poured into a glass tube (diameter 10 mm, length 120 mm, respectively) the bottom of which was equipped with a porous disc. The height of the packing was 100 mm.

0.1–0.3 ml of the reaction mixture from the chloramine T labelling procedure was placed on the top of the bed just as the last few drops of the distilled water, in which the gel was allowed to be settled, soaked into the bed. The sample solution, containing 1–10 μg of iodothyronine, was allowed to soak into the gel. Within 10–20 min iodothyronines from the sample solution were adsorbed on the top of the gel. Free radioiodine and reagents (i.e. chloramine T, phosphate buffer and sodium metabisulphite) were eluted with three-four column volume of distilled water. This procedure did not result in any displacement of the adsorbed iodothyronines from the top of the gel.

Aqueous solution of ethyl alcohol was used as eluent the pH of which was adjusted with citrate or veronal buffer over the pH range 4–12.5 to the required value. A peristaltic pump delivered the eluent at a flow rate of 22–25 ml/hour.

The same glass chromatographic column was used for all experiments reported here. The radioactivity of the effluent was monitored with a scintillation counting system and the count rate was recorded by an x–y plotter.

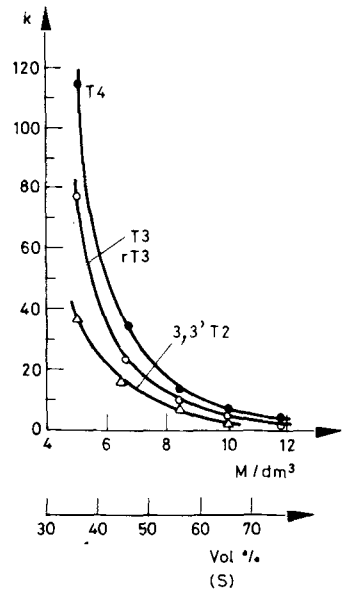

Fig. 1. The dependence of the distribution coefficient of 3,3'T2, T3, rT3 and T4 on the ethanol concentration at pH = 4

Evaluation of the experimental results

Since Sephadex LH-20 dextran gel was used in our case as adsorbent and no gel filtration of iodothyronines was observed, the distribution coefficient was calculated according to Eq. (1), used in adsorption chromatography:[8]

$$k = \frac{V_e - V_o}{V_t - V_o} \tag{1}$$

where V_e, V_t and V_o stands for the elution volume of the solute, the total volume of the gel bed and for the void volume, respectively.

Results and discussion

The dependence of the distribution coefficient on the ethyl alcohol concentration of the eluent at pH ≈ 4

Fig. 1 shows the k vs. (S) plots for T2, T3, rT3 and T4. The same results are shown in a lg – lg plot in Fig. 2.

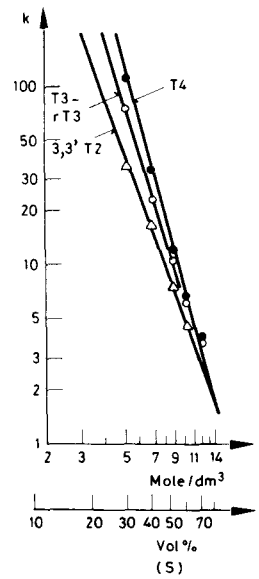

Fig. 2. The data of Fig. 1. shown in lg–lg plot

Elution performed by the use of aqueous solution of ethyl alcohol can be attributed to the competition existing between sample and solvent molecules in the liquid phase for a place on the adsorbent surface:[8]

$$Dx{-}T + n.S \rightleftharpoons Dx{-}S_n + T \tag{2}$$

where Dx stands for the dextran gel, T for the iodothyronine in question and S for the solvent.

Expressing the equilibrium constant (K) of reaction (2):

$$K = \frac{(Dx{-}S_n)\,(T)}{(Dx{-}T)\,(S)^n} = \frac{1}{k} \cdot \frac{(Dx{-}S_n)}{(S)^n} \tag{3}$$

and neglecting the change of the term $(Dx{-}S_n)$ as well as introducing k^o (i.e. the distribution coefficient at 1 mole/l solvent concentration), the distribution coefficient can be expressed as follows:

$$\lg k = \lg k^o - n \cdot \lg (S) \tag{4}$$

The linearity of the lg k vs. lg (S) plots shown in Fig. 2 is in good agreement with Eq. (2).

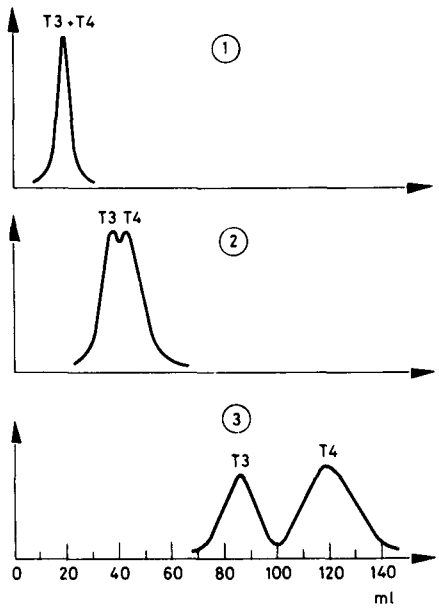

Fig. 3. Elution curves obtained by the co-chromatography of T3 and T4. Curve 1 – eluent 50 vol% ethanol, pH = 4, curve 2 – eluent 40 vol% ethanol, pH = 4, curve 3 – eluent 30 vol% ethanol, pH = 4

As it turns out from Eq. (4) the k values of the iodothyronines can be controlled by varying the ethyl alcohol concentration of the eluent. On the basis of Eq. (4) the influence of the ethyl alcohol concentration on the selectivity (α) can be calculated as follows:

$$\alpha = \frac{k_2}{k_1} = \frac{k_2^o}{k_1^o} \cdot (S)^{n_1 - n_2} \qquad (5)$$

Fig. 3 shows a few elution patterns representing the influence of the ethyl alcohol concentration on the separation of T3 and T4, while Fig. 4 shows the dependence of the selectivity (α) of the separation of rT3 and 3,3'T2 on the ethyl alcohol concentration.

It should be noted that at pH $\approx$ 4 iodothyronines with the same number of iodine atoms per molecule (e.g. T3 and rT3) can not be separated by varying the organic solvent concentration of the eluent. Such a separation can be accomplished by increasing the pH of the eluent to 6–6.5, i.e. where the dissociation of the adsorption affinities of T3 and rT3 due to the different pK_{OH} values takes place.

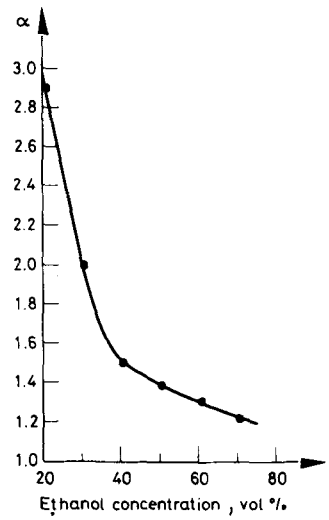

Fig. 4. The dependence of the selectivity coefficient on the ethanol concentration of the eluent for the separation of rT3 and 3,3'T2

From practical point of view the usefulness of Eq. (4) lies in that by the use of it the eluting power of the aqueous ethyl alcohol eluent of any concentration can be calculated provided the k^o and n values are known. These latter are enlisted in Table 2 for the four iodothyronines investigated.

The explanation of the physical meaning of the n values is out of the scope of this paper. It should be, however, noted that according to the molecular adsorption model proposed by SOCZEWINSKI the slope of the lg k vs. lg (S) plot, i.e. the n value, should represent the number of solvent molecules displaced from the adsorbent surface by a single solute molecule.[9,10]

The finding that the higher the number of iodine atoms per molecule the greater n and k^o leads to the conclusion that besides the unionised phenolic hydroxyl group the iodine substituents of the iodothyronines can also interact with the dextran gel.

Table 2
The k^o and n values obtained by linear extrapolation of the log k vs. log (S) plots of Fig. 2

Iodothyronine	3,3'T2	T3	rT3	T4
k^o	2 500	10 000	10 000	40 000
n	2.95	3.45	3.45	3.98

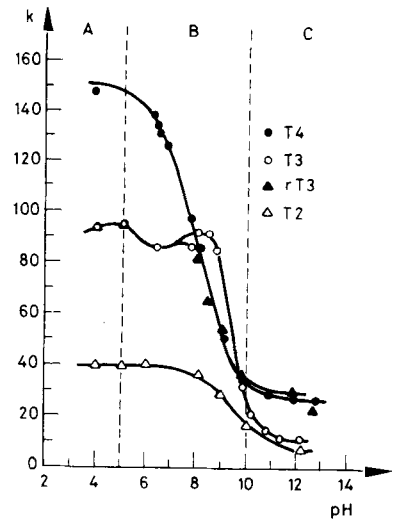

Fig. 5. The pH dependence of the distribution coefficient of 3,3'T2, T3, rT3 and T4 at 30 vol% ethanol concentration

Influence of the pH on the distribution coefficient

As it can be concluded from the data of Table 1, with increasing pH of the eluent first the phenolic hydroxyl group of the 3',5'-substituted iodothyronines (T4, rT3 and 3',5'T2), then that of the 3' substituted derivatives (3,3',5T3; 3'Tl) and next that of iodothyronines with no iodine atom at all in the outer ring (3Tl, 3,5T2) will be ionised.

If the adsorption of iodothyronines on dextran gel would be influenced by the ionisation of the phenolic hydroxyl group only, an elution order could be expected which corresponds to the sequence of the increasing pK_{OH} values. In effect, the adsorption affinity depends on the number of iodine atoms per molecule as well (i.e. the greater the number of iodine atoms per molecule the higher the distribution coefficient) which render the elution order more complicated as compared to organic acids bearing no halogen substituents.

For the interpretation of the pH dependence of the distribution coefficients, the whole pH range is advantageously to be divided into three parts, labelled with A, B and C in Fig. 5.

In the pH range 4–5 (A in Fig. 5) little variation, if any, of k with the pH can be observed. The elution order corresponds to the increasing number of iodine atoms per molecule, i.e. first T2, then T3 and rT3 and next T4 is eluted. The

two iodothyronines, bearing three iodine atoms alike (T3 and rT3), exhibit the same distribution coefficient.

In the pH range 5–10 (B in Fig. 5), first the decrease of the k value of T4 due to the ionisation of the phenolic OH group can be observed.

This is followed by the drop of the distribution coefficient of rT3 and about two pH units later that of T3 as well. In the narrow pH range 8–9 the reversal of the elution order of T3 and T4 can be observed.

At pH > 10 (C in Fig. 5) the phenolic hydroxyl group of any iodothyronines is ionised and consequently the elution order will depend again on the number of iodine atoms per molecule only, i.e. the elution order observed at pH ≈ 4 will be restored. The only exceptions are T3 and rT3: in spite of the fact that both iodothyronines contain three iodine atoms alike, the distribution coefficient of rT3 exceeds considerably that of T3.

From the experimental results reported here the conclusion can be drawn that by choosing the proper pH and ethanol concentration of the eluent the four radioiodine labelled iodothyronines (T4, T3, rT3 and 3,3'T2) can be separated using LH-20 dextran gel as adsorbent. The radioiodine labelled iodothyronines are unstable in aqueous solution, they are to be stabilised with organic solvents like ethanol.[11] Since in our case the elution is performed with aqueous ethanol solution the effluent can be used without any additional treatment for in vitro diagnostic purposes. The fact that the iodothyronines separated are during the separation process in aqueous ethanol solution, minimized the decomposition during the separation process as well.

References

1. M. C. PATEL, K. B. DESAI, M. N. MEHTA, H. I. GLENN, J. Appl. Radiation Isotopes, 25 (1974) 421.
2. J. WEEKE, H. ÖRSKOV, Scand. J. Clin. Lab. Invest., 32 (1973) 357.
3. A. J. W. BROOK, S. HOUSLEY, J. Chromatog., 42 (1969) 112.
4. H. M. ORTNER, B. E. SCHREIBER, H. SPITZY, Z. Anal. Chem., 252 (1970) 260.
5. G. TÓTH, Radiochem. Radioanal. Letters, 29 (1977) 207.
6. G. TÓTH, Radiochem. Radioanal. Letters, 30 (1977) 297.
7. F. BLASI, R. V. MASI, J. Chromatog., 28 (1967) 33.
8. L. R. SNYDER, Principles of Adsorption Chromatography, Marcel Dekker, New York, 1968.
9. E. SOCZEWINSKI, Anal. Chem., 41 (1969) 179.
10. E. SOCZEWINSKI, W. GOLKIEWICZ, Chromatographia, 4 (1971) 501.
11. J. M. KJELD, S. F. KUKU, L. DIAMANT, T. R. FRASER, G. F. JOPLIN, K. MASHITER, Clin. Chim. Acta, 61 (1975) 381.