*Journal of Radioanalytical and Nuclear Chemistry, Articles, Vol. 121, No. 1 (1988) 17–27*

# A GENERAL APPROACH TO THE CHROMATOGRAPHIC BEHAVIOUR OF $^{125}$I-LABELLED IODOTHYRONINES AND TYROSINE METHYL ESTER DERIVATIVES OF STEROIDS

G. TÓTH

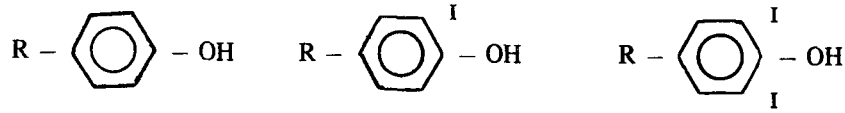
*Institute of Isotopes of the Hungarian Academy of Sciences, H-1525 Budapest, P.O.B. 77 (Hungary)*

(Received August 11, 1987)

The separation of low molecular weight compounds labelled with $^{125}$I has gained importance with the advent of radioimmunoassay (RIA) in which overwhelmingly $^{125}$I-labelled analytes are used as tracers. The chloramine T labelling method proved to be the most suitable procedure to introduce radioiodine atom via aromatic electrophilic substitution either in the thyronine molecule or in the tyrosine methyl ester (TME) side chain coupled as a prosthetic group to different compounds which do not possess phenolic hydroxyl groups.

## Introduction

In our previous papers we reported studies on the adsorption chromatographic behaviour of iodothyronines,[1-3] iodotyrosines,[4] steroids,[5-8] and prostaglandins[9] to which the tyrosine methyl ester (TME) side chain was coupled. Our primary aim consisted in the development of a separation method which enables the recovery of a single labelled compound (i.e. the tracer) from the labelling reaction mixture with high specific activity and radiochemical purity. All of the other components, like unlabelled starting material, double-labelled and unidentified labelled products as well as unreacted free radioiodine are considered as by-products which cannot be used in RIA. Since two iodine atoms can be introduced into the phenyl ring at the o and/or o' positions relative to the phenolic hydroxyl group, the simultaneous formation of mono- and double-labelled products is to be taken into account. The following scheme illustrates the composition of the Chloramine T labelling mixture. (The inactive components of the labelling procedure e.g. Chloramine T reduced by sodium metabisulfite etc. are not listed).




Starting material | Monoiodo labelled product | Diiodo labelled product



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*Akadémiai Kiadó, Budapest*

R stands – in case of iodothyronines – for the phenylalanine diphenyl ether residue while – in case of steroids – for the O-carboxymethyloxime TME or hemisuccinate TME side chain.

Taking into account that either the mono- or the double-substituted phenyl derivatives are used as tracer in radioimmunoassay, the separation of the labelling mixture is to be focused on the recovery of either the mono- or the double-labelled product. On the other hand, the non-labelled starting material is to be quantitatively separated from the tracer, otherwise it lowers the specific activity of the tracer drastically. From the scheme it turns out that the method to be applied for the separation of $^{125}$I-labelled tracer should meet the requirement to distinguish the starting material and its mono- and disubstituted derivatives.

Albeit for micro-scale preparation of $^{125}$I-labelled tracers several authors have reported successful application of paper and thin layer chromatography, for large scale production, when high activities are to be processed, elution from paper or thin layer may raise difficulties. Thus for the separation of high activity tracers only column chromatography seems to be the method of choice.

The structural similarity of the starting material, the mono- and double-labelled products may raise difficulties especially when the tyrosine methyl ester (TME), into which radioiodine is incorporated, is coupled to a larger molecule as a prosthetic group. In case of steroid carboxymethyloxime TME derivatives, e.g. the introduction of an iodine atom increases the molecular weight by about 5% only.

To reconcile the adverse requirements of high purity and high radioactive concentration (i.e. minimum band broadening) of the tracer the optimum resolution is to be adjusted. To do so, the most successful way is to optimize the separation by choosing an eluent of proper strength. If the choice of the eluent and in case of a binary mixture that of the relative proportion of the components is made empirically, it may require considerable preliminary investigations.

Eluotropic series like those published by TRAPPE, STRAIN, JACQUES and MATHIEU may assist to select the most proper solvent as eluent,[10–11] however, they do not allow the precise adjustment of the required distribution coefficient of the solute. For polar adsorbents like silica and magnesia the eluent strength parameter introduced and defined by SYNDER[12] makes the adjustment of the required k value possible.

For nonpolar adsorbents (charcoal, polyamide) there are less complete eluotropic series available and especially for dextran gel no data in this respect exist at all. Since in case of dextran gel adsorbents no pure solvents can be used as eluents the required resolution is usually achieved by the use of water-organic solvent binary eluents.[1–2]

The aim of this paper is to demonstrate that the distribution coefficient (k) of several solutes varies with the organic solvent concentration of the water/ethanol

eluent (X) according to

$$\log k = \log k_0 - n \log X \qquad (1)$$

which makes the adjustment of the distribution coefficient possible by the proper choosing of the eluent composition.

## Experimental

Thyronine, iodothyronines, steroid with aromatic A-ring (estriol) and steroids to which TME prosthetic group was coupled were labelled with $^{125}I$ by the use of the chloramine T method, as was described previously,[1-9]

An SR/50 Pharmacia chromatographic column (I. D. 10 mm) equipped with a thermostat jacket was filled with Sephadex LH-20 dextran gel swollen in distilled water or citrate buffer (pH 4) prior to being packed in the column. The height of the packing was 100 mm. The sample – containing either the authentic labelled compound or an aliquot of the chloramine T labelling mixture – was placed in 0.1–0.3 ml on the top of the column and allowed to soak; 10–20 minutes later, i.e. when adsorption equilibrium had been attained, elution was performed with ethanol-water. The effluent was passed over a NaI/Tl scintillation crystal and the count rate was monitored by a rate meter and registered by an x–y plotter. When $^{3}H$-labelled analytes were chromatographed, the effluent was collected with a fraction collector and its radioactivity was determined by a liquid scintillation counter. A peristaltic pump, flow rate 22–24 ml/h, delivered the eluent. The distribution coefficient was calculated according to

$$k = \frac{V_e - V_o}{W} \qquad (2)$$

where $V_e$, $V_o$ and W stand for the elution volume, the dead volume and the weight of the adsorbent, respectively.

## Results

### *Iodothyronines*

To elucidate the effect of iodine substituents on chromatographic behaviour the family of iodothyronines along with thyronine itself proved to be a useful model. Namely at most four iodine atoms can be incorporated into the diphenyl ether

skeleton at positions 3,5 and 3', 5'. Thus altogether eight mono-, di-, tri- and tetra-substituted thyronines may exist, out of them four are isomers (T3–rT3, 3T1–3'T1, 3, 5T2–3, 3'T2–3', 5'T2).

Via aromatic electrophilic substitution radioiodine can only be introduced into positions 3' and/or 5', i.e. into the o, o' positions with respect to the phenolic hydroxy group. The latter can influence the adsorption of iodothyronines drastically; the ionization of the phenolic OH practically cancels the adsorption of the whole molecule.[1] On the other hand, the ionization is governed by the $pK_{OH}$ which is 9, 8.3 and 6.3 for phenols, iodophenols and diiodophenols, respectively.

At pH 4, i.e. when the phenolic hydroxyl is unionized, the log k vs, log X plot of the investigated five iodothyronines is linear (Fig. 1). From the plots shown in Fig. 1 several conclusions can be drawn. First, the distribution coefficient increases with increasing number of iodine atoms per molecule, consequently the lowest adsorption

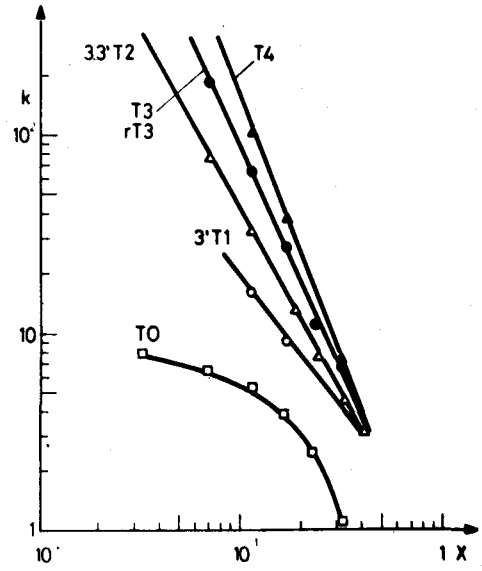


Fig. 1. Log k vs. log X plot of thyronine (TO), 3-iodothyronine (3'T1), 3, 3'-diiodothyronine (3,.3'T2), 3, 3', 5-triiodothyronine (T3), 3, 3', 5'-triiodothyronine (rT3) and thyroxine (T4)

affinity towards the dextran gel can be observed in case of tritium-labelled thyronine. In addition, because of the weak adsorption of thyronine its log k vs. log X plot considerably deviates from linearity. On the other hand T3 and rT3, with three iodine substituents per molecule, behave identically. Since the log k vs. log X plots shown in Fig. 1 spread fanwise with decreasing ethanol concentration in the eluent the

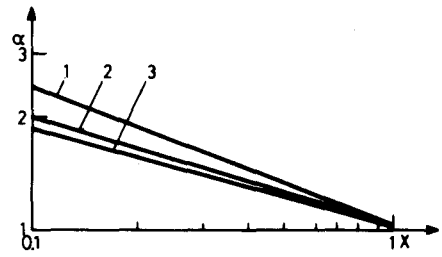


Fig. 2. Selectivity of the separation as a function of the ethanol concentration of the eluent; curves; $1 - \alpha = \frac{k_{3,3'T2}}{k_{3'T1}}$, $2 - \alpha = \frac{k_{T3}}{k_{3,3'T2}}$, $3 - \alpha = \frac{k_{T4}}{k_{T3}}$

selectivity of separation increases when lowering the ethanol concentration (Fig. 2.). When substituting the actual values of $k_o$ and n into Eq. 1 the following equations are obtained:

$$\log k = -0.01 - 1.3 \log X \quad (T1) \tag{3}$$
$$\log k = -0.19 - 1.8 \log X \quad (T2) \tag{4}$$
$$\log k = -0.26 - 2.2 \log X \quad (T3) \tag{5}$$
$$\log k = -0.26 - 2.2 \log X \quad (rT3) \tag{6}$$
$$\log k = -0.38 - 2.6 \log X \quad (T4) \tag{7}$$

The selectivity of the separations vs. log X plots can thus be calculated according to

$$\alpha = \frac{k_n}{k_{n-1}} = \frac{k_o^n}{k_o^{n-1}} X^{n_1 - n_2} \tag{8}$$

i.e. log α depends linearly on log X (Fig. 2.).

*Steroids*

For several steroids and $^{125}$I-steroid derivatives similarly linear log k vs. log X relationships were obtained. The log k vas. log X plots of tritium-labelled cortisol, progesterone, testosterone, estriol, estrone, and 17 beta-estradiol are shown in Fig. 3. In case of progesterone, deviations from the linearity can be observed at low ethanol concentration but, all of the other plots obey Eq. (1). Substituting the $k_o$ and n

values into Eq. (1), the following relationships are obtained:

| | | |
|---|---|---|
| cortisol | $\log k = -0.08 - 0.41 \log X$ | (9) |
| progesterone | $\log k = -0.48 - 1.6 \log X$ | (10) |
| testosterone | $\log k = -0.53 - 1.74 \log X$ | (11) |
| estriol | $\log k = -0.30 - 1.65 \log X$ | (12) |
| estrone | $\log k = -0.65 - 1.75 \log X$ | (13) |
| 17-beta-estradiol | $\log k = -0.5 - 2.1 \log X$ | (14) |

Inspection of the plots in Fig. 3 reveals that the log k vs. log X relationship obeys Eq. (1) with the only exception of that of progesterone; in this case at low ethanol concentration deviation from the linearity can be observed. Like in case of iodothyronines the plots spread fanwise with the dilution of the ethanol.

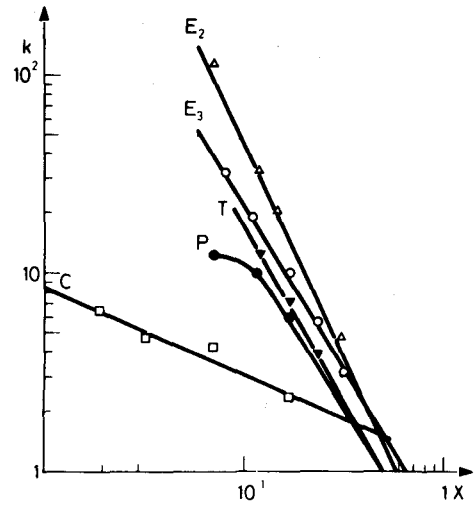


Fig. 3. Log k vs. log X plot of $^{3}$H-labelled cortisol (C), progesterone (P), testosterone (T), estriol (E3) and 17-β-estradiol (E2)

The introduction of $^{125}$I-atom into the steroid molecule either into the aromatic A-ring (e.g. into estriol, estradiol or estriol) or into tyrosine methyl ester coupled via carboxymethyloxime or succinyl prosthetic group increase the elution volume and thus the distribution coefficient as well. The dependence of log k on log X for $^{125}$I-substituted steroids are as follows:

| | | |
|---|---|---|
| Cortisol-3-CMO-3-iodo-TME | log k = 0.02 – 1.36 log X | (15) |
| Progesterone-11-succinyl-3-iodo-TME | log k = –1.3 – 2.7 log X | (16) |
| Testosterone-3-CMO-3-iodo-TME | log k = –1.16 – 3.1 log X | (17) |
| Testosterone-3-CMO-3, 5-diiodo-TME | log k = –0.92 – 3.1 log X | (18) |
| Estriol-6-CMO-3-iodo-TME | log k = –0.52 – 2.3 log X | (19) |
| 2/4-iodoestradiol | log k = –0.69 – 2.4 log X | (20) |
| 17-β-estradiol-6-CMO-3-iodo-TME | log k = –0.36 – 2.1 log X | (21) |
| 2/4-iodo-17-β-estradiol | log k = –0.37 – 2.2 log X | (22) |

From the comparison of Eqs (9) to (14) and Eqs (15) to (22) several conclusions can be drawn. First of all it can be stated that the slope of the log k vs. log X plot is higher in case of $^{125}$I-substituted derivatives as compared with the tritium-labelled parent molecules, i.e. the carboxymethyloxime tyrosine methyl ester or succinyl tyrosine methyl ester prosthetic group and/or the iodine substitutent increase the adsorption affinity. Inspection of the log k vs. log X plots of testosterone,

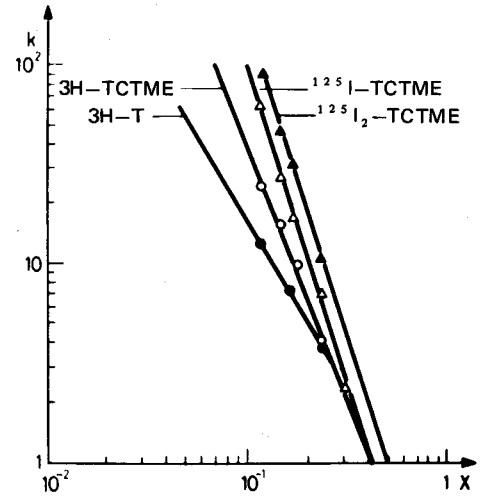


Fig. 4. The log k vs. log X plot of $^{3}$H-labelled testosterone ($^{3}$H–T), $^{3}$H-labelled testosterone-3-O-carboxymethyl) oxime tyrosine methyl ester ($^{3}$H–TCTME), testosterone-3-(O-carboxymethyl) oxime 3-iodo-tyrosine methyl ester ($^{125}$I–TCTME) and testosterone-3-(O-carboxymethyl)oxime 3,5-diiodo tyrosine methyl ester ($^{125}$I$_2$-TCTME).

testosterone-3-CMO-TME and testosterone-3-CMD-iodo-TME shown in Fig. 4 reveals that both the prosthetic group and the iodine substitutent contribute to the increase of the slope and consequently the adsorption affinity towards the gel. The

log k vs. log X plot of testosterone-3-CMO-3, 5-diiodo-TME, also presented in Fig. 4 indicates that the introduction of the second iodine atom into the TME residue increases only $k_o$ and does not increase the slope, i.e. n.

## Discussion

The experimental results reported in this paper support Eq. (1) which can be derived assuming that the eluent consists of an active and inert solvent and the elution of the solute takes place due to its displacement caused by n active solvent molecules:

$$D - S + n\,X \rightleftharpoons D - X_n + S \tag{23}$$

where S and X stand for the solute and the active solvent in the mobile phase, while D – S and D – X in the stationary phase, i.e. adsorbed on the dextran gel.

Strong and reversible interactions between solute and adsorbent may result in the formation of molecular complexes characterized by the stability constant (K) derived from the law of mass action

$$K = \frac{(D - X_n)\,(S)}{(D - S)\,(X)^n} \tag{24}$$

where the bracketed terms mean the activities expressed in mole fraction. From Eq. (24), the distribution coefficient (defined as $k' = (D - S)/(S)$ can be expressed as follows:

$$\log k' = -\log K + \log (D - X_n) - n \log (X) \tag{25}$$

Introducing the distribution coefficient (k) (defined as $m/C_m$ where m is the mass of solute retained per unit mass of stationary phase and $C_m$ is the concentration of solute in mobile phase) we obtain:

$$k = \frac{V_e - V_o}{W} = k' \frac{W}{V_s}$$

$$\text{and } \log k = -\log K + \log (D - X_n) + \log \frac{W}{V_s} - n \log X \tag{26}$$

Reducing the constant terms in $k_o$ the physical meaning of which is the distribution coefficient in pure solvent, Eq. (26) can be simplified as follows:

$$\log k = \log k_o - n \log X. \tag{27}$$

Since $(D - X_n)$ varies within a very limited range provided X is not extremely low, it can be considered as constant and thus can be incorporated into the term $k_o$.

The effect of the composition of the eluent on the distribution coefficient has been investigated by several authors mostly in case of polar adsorbents like silica, alumina and magnesia. SNYDER characterized the elution power of organic solvents on theoretical basis taking into account the effect of molecular size and structure as well as the type and composition of the eluent.[12] Later SOCZEWINSKI and GOLKIEWICZ[13,14] have elaborated a molecular model of adsorption on silica.

In both cases it is supposed that an active solvent is diluted with an inert one which is not adsorbed on the stationary phase. Thus it can be assumed that the molecules of the solute and the active solvent displace one another.

Linear log k vs. log X relationships can also be derived when the solute-adsorbent and solvent-adsorbent interactions are taken into account separately and the competitive formation of the adsorption complexes is described by the law of mass action as was done by SOCZEWINSKI and GOLKIEWICZ.[13,14] Assuming that the molecules of the solvent and solute displace one another in a 1:1 ratio, the slope of the log k vs. log X plot should be 1. In case of solutes possessing more than one active group capable of interaction with the adsorbent the slope would be equal to the number of active groups of the solute according to the simplified model. The simplification lies in the neglect of the solute-solute, solvent-solvent and adsorbed solute-solvent interactions which may alter the slope of the log k vs. log X plot, i.e. the absolute value of n. Even assuming that these interactions do not exist at all, the size and configuration of the solute with more than one active group may also disturb the ideal case. Inspecting the n values obtained for iodothyronines summarized in Table 1, it can be concluded that in case of Sephadex LH-20 adsorbent n increases with increasing number of the active groups, i.e. phenolic hydroxy and iodine substituent (s). Nevertheless, the outer (phenolic) ring and the inner (alanine side chain) ring are inclined at an angle of about 120 degrees and the two rings can rotate around the ether linkage. The equality of n for T3 and rT3 speaks for the fact that both phenyl rings exhibit the same adsorption affinity towards the gel. This can only be explained by the penetration of the iodothyronines into the gel structure. In case of silica based nonpolar stationary phase ($\gamma$-Bondapak $C_{18}$) the adsorption of the solute is restricted to the surface which precludes the simultaneous adsorption of both rings.[15] Since the charged carboxy and amine groups near the inner ring counteract the

adsorption, it is the outer ring which governs the adsorption of the whole molecule. Therefore the elution of T3 which is monoiodinated in the outer ring precedes that of rT3, exhibiting diiodinated outer ring. In contrast to γ-Bondapak $C_{18}$ in case of Sephadex LH-20 adsorbent, T3 and rT3 are eluted simultaneously, which means that the n values do not differ at all, provided the pH is low enough (pH 4) to prevent the dissociation of the phenolic hydroxy. The log k vs. log X plot of thyronine shown in Fig. 1 supports the finding of DETERMANN and WALTER,[16] according to which, compounds with unionized phenolic hydroxy groups may interact with the dextran gel (probably with the hydroxy-ether group used to cross-link the dextran chains) resulting in distribution coefficients higher than the unity. Nevertheless the log k vs. log X plot is non-linear just in case of thyronine, which can be attributed to the fact that in this case the water cannot be considered as inactive component of the binary eluent as it can be in case of strongly adsorbed iodothyronines. The inspection of the log k vs. log X plots of the five iodothyronines reveals complete agreement with the finding of BROOK and HOUSLEY[17] as well as BLASI and MASI,[18] according to which, iodine substituents greatly increase the elution volume and thus the distribution coefficient as well. Nevertheless the finding that n in Eq. 1 is not integer indicates that the solvent-solvent and solute-solute interactions cannot be precluded, as was done in case of Eq. 1. It is worth mentioning that the LH-20 gel cannot distinguish T3 and rT3 which possess three iodine atoms per molecule alike. Their separation can be performed by making use of their different $pK_{OH}$.[3]

In spite of the fact that n is not an integer, in case of iodothyronines it is proportional to the number of active groups (phenolic hydroxy + iodine atoms) per molecule, which speaks for that n is not equal but proportional to the number of solvent molecules which displace one solute molecule.

There are several differences in the chromatographic behaviour of thyronines and steroids or steroid-TME derivatives. As can be seen from the example of testosterone (Fig. 4), the 3-CMO-TME prosthetic group results in a considerable increase of n, which indicates that the prosthetic group contributes to the adsorption affinity. Further increase of n can be observed when a radioiodine atom is introduced into the TME residue. Surprisingly, the incorporation of the second iodine atom into the TME side chain does not increase the slope of the log k vs. log X plot. This is clearly illustrated by Fig. 4 which presents the log k vs. log X plots of testonsterone-3-CMO-TME and its 3-iodo as well as 3, 5-diiodo derivatives. From the fact that the incorporation of one or two iodine atom(s) into the TME residue does not increase n considerably (as compared to the iodothyronines) the conclusion can be drawn that it is the steroid skeleton which is mainly responsible for the adsorption on the gel and the 3-iodo or 3, 5-diiodo side chain has in this respect a minor effect.

Evidence that in case of steroid-CMO-TME derivatives neither the CMO-TME side chain nor the iodine substituent(s) introduced into the 3/5 positions of the TME residue play a significant role in the adsorption process can be obtained from the pH dependence of the distribution coefficient. As was demonstrated in case of iodothyrosines[4], iodothyronines[2] and diethylstilbestrol[19] the ionization of the phenolic hydroxy practically cancels the adsorption of the whole molecule. On the contrary the ionization of the phenolic hydroxy of the TME coupled to steroids results only in a slight decrease of the distribution coefficient, indicating the negligible contribution of the iodine substituted TME residue to the adsorption affinity as compared to the steroid skeleton itself. The adsorption energy change caused by ionization of the hydroxy group will be reported in a forthcoming paper.

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