A GENERAL METHOD FOR THE PRODUCTION OF ^{125}I-LABELLED LOW-MOLECULAR-WEIGHT TRACERS FOR RADIOIMMUNOASSAY

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Abstract

A GENERAL METHOD FOR THE PRODUCTION OF ^{125}I-LABELLED LOW-MOLECULAR-WEIGHT TRACERS FOR RADIOIMMUNOASSAY.

A general method is described for the separation of low-molecular-weight ^{125}I-labelled tracers from a chloramine-T labelling mixture, containing the starting material, the tracer, labelled by-products, free radioiodine and reagents used in the labelling procedure. Small molecules like tyrosine, thyronine and steroids as well as their tyrosine methyl ester conjugates are adsorbed on Sephadex LH-20 dextran gel. The introduction of iodine atoms increases the elution volume and the distribution coefficient in proportion to the number of iodine atoms per molecule. Organic solvents like ethanol are also adsorbed on dextran gel, which results in desorption of the starting material or its iodine-substituted derivatives. The elution order follows the number of iodine substituents per molecule, i.e. unsubstituted starting material, mono-, di-, tri- and tetra-substituted molecules. Using a water/ethanol binary eluent in which water can be considered as inert diluent, the elution is governed by the ethanol concentration. The finding that the logarithm of the distribution coefficient of the solutes is linearly correlated with the logarithm of the ethanol concentration permits an optimization of the separation.

1. INTRODUCTION

In the past decade, low-molecular-weight tritium-labelled tracers have been gradually replaced by ^{125}I-labelled tracers. Tyrosine and thyronine were labelled from the outset with ^{125}I as they represent the only group of biologically active compounds which originally contain one or more iodine atoms. It has also been found that small molecules, like steroids and prostaglandins, which do not contain phenolic hydroxyl (which would enable aromatic electrophilic substitution of iodine), can be labelled with ^{125}I when a prosthetic group like tyrosine methyl ester (TME) is coupled to them.

The ^{125}I-labelled tracers have to meet the following requirements: (a) high specific activity, (b) high chemical and radiochemical purity, and (c) long shelf-life.

The quality of the tracer depends on both the labelling procedure and the separation method applied for purifying the tracer.

A great many radioiodination methods, such as the chloramine-T, enzymatic, electrolytic and iodogen methods, are available. Their common feature is that all of them convert radioiodine to electrophilic reagents (e.g. I^+, H_2OI^+) which may attack the positions 3 and/or 5 of tyrosines or the positions 3′ and/or 5′ of thyronines. At these positions, both aromatic electrophilic substitution and isotopic exchange may take place. For the production of a tracer of high and controlled specific activity, only the substitution reaction can be taken into account. In this case, when carrier-free ^{125}I is used in the labelling procedure, the specific activity of the tracer approaches 2180 Ci/mmol (81 TBq/mmol). This specific activity can be achieved via isotopic exchange only if the amount of the compound to be labelled is infinitesimally small as compared with the amount of radioiodine. Since this condition cannot be fulfilled, the specific activity varies within a wide range, governed by the stoichiometry and, in addition, by the reaction conditions.

After quenching the labelling reaction, the reaction mixture contains the starting material, the tracer, labelled by-products, free radioiodine and reagents used in the labelling procedure.

The strategy of tracer purification depends on whether the tracer contains (a) one radioiodine atom at position 3 or 3′, or (b) one iodine atom at position 3 or 3′ and one radioiodine atom at position 5 or 5′.

(a) Besides 3-iodotyrosine, all steroids and prostaglandins to which the TME prosthetic group is coupled belong to the first group, in which 3-radioiodo-

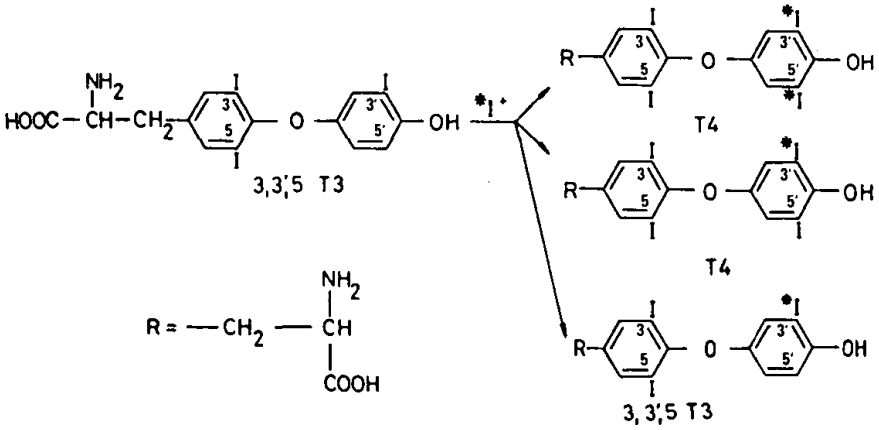

FIG. 1. Production of ^{125}I-T4 from T3.
Left: 3,3′, 5-triiodo-L-thyronine (T3); right: single- and double-labelled thyroxine formed via aromatic electrophilic substitution and isotopic exchange, and single-labelled 3,3′, 5-triiodo-L-thyronine formed via isotopic exchange.

tyrosine or 3,5-diradioiodotyrosine or TME derivatives are simultaneously formed from the starting material. Since the 3,5-diiodo-TME conjugates usually lack immunoreactivity, these are to be considered as labelled by-products and only 3-radioiodo-TME conjugates can be used as tracers. On the other hand, the antibody raised against the TME conjugates cross-reacts with the starting material. The formation of the double-labelled (i.e. 3,5-diradioiodo) TME derivative can be suppressed when the starting material is used in great excess compared with radioiodine; however, in this case the excess of the starting material drastically decreases the specific activity of the tracer. When no excess of starting material is used, tracer purification must focus on the separation of the tracer and the double-labelled by-product. With an excess of starting material, complete separation of the tracer from the starting material can be achieved.

(b) When the analyte in the radioimmunoassay contains two iodine atoms at 0,0′ positions relative to the phenolic hydroxyl group (e.g. 3,5t2, 3′,5′T2, rT3, T4), three labelled products are formed from the starting material. This is illustrated in Fig.1, which shows the production of ^{125}I-T4 from T3.

Of the components in Fig.1, the low-specific-activity ^{125}I-T3 formed via isotopic exchange and the starting material T3 cannot be separated from each other, as is also the case for single- and double-labelled T4. Increasing the amount of T3 compared with ^{125}I, the formation of the double-labelled T4 can be suppressed [1]. Thus the labelling mixture will contain low-specific-activity ^{125}I-T3 and high-specific-activity, single-labelled T4, which can easily be separated.

Many purification procedures have been used for separating ^{125}I-labelled low-molecular-weight tracers, for instance paper chromatography, thin-layer chromatography, ion-exchange chromatography, adsorption chromatography and high-pressure liquid chromatography. However, for large-scale production and high activities, only column chromatography seems to be the proper separation method. If the chromatography aims at preparative separation of the ^{125}I-labelled tracer, only the resolution related to two-band pairs, i.e. those of the tracer and the adjacent bands, is of major concern. To reconcile the adverse requirements of high purity and high radioactive concentration of the tracer (i.e. minimum band broadening), the optimum resolution of the separation has to be adjusted. This can be achieved only when the elution of the tracer, the starting material and the labelled by-products can be adjusted to the optimum value by choosing the proper eluent strength. This is usually achieved by trial and error, using binary or ternary systems. However, considerable preliminary investigations are necessary for this and exact adjustment of the eluent strength is not possible.

We aim at demonstrating that the separation of the tracer can be performed with a water/organic solvent binary eluent, the strength of which can be adjusted to any required value by varying the concentration of the organic solvent.

2. MATERIALS AND METHOD

The tritium-labelled progesterone (P), testosterone (T) and estriol (E3) used are products of Amersham. The ^{125}I-labelled compounds were produced with the chloramine-T method [2] from the following starting materials:

- L-tyrosine (t0)
- 3-iodo-L-tyrosine (t1)
- 3,5-diiodotyrosine (t2)
- L-thyronine (T0)
- 3′-iodo-L-thyronine (3′T1)
- 3,3′-diiodo-L-thyronine (3,3′T2)
- 3,3′, 5-triiodo-L-thyronine (T3)
- 3,3′, 5′-triiodo-L-thyronine (rT3)
- 3,3′, 5,5′-tetraiodo-L-thyronine (thyroxine) (T4)
- estriol (E3)
- progesterone-11-succinyl-tyrosine methyl ester (PTME)
- testosterone-3-/O-carboxymethyl/oxime tyrosine methyl ester (TTME)
- estriol-6-/O-carboxymethyl/oxime tyrosine methyl ester (ETME).

The apparatus and adsorbent used are described in Refs [3, 4]. An SR/50 Pharmacia chromatographic column, equipped with a thermostat jacket (i.d. 10 mm), was filled with Sephadex LH-20 dextran gel swollen in citrate buffer (pH 4) before being packed into the column. With a peristaltic pump the effluent was passed through the column over a NaI-Tl scintillation crystal and the count rate was monitored by a rate meter and registered by an x-y plotter. The chloramine-T labelling mixture (0.1–0.4 mL) was placed on top of the column and was allowed to soak in. After adsorption equilibrium had been attained (10–20 min) the elution was started with a flow rate of 22–24 mL/h. The pH of the eluent was adjusted to the required value with citrate buffer. The temperature of the column was kept at 25°C with an ultrathermostat connected to the jacket of the column.

The distribution coefficient of the ^{3}H- and ^{125}I-labelled compounds was calculated according to

$$k = \frac{V_e - V_0}{W} \qquad (1)$$

where V_e, V_0 and W stand for the elution volume, the dead volume and the weight of the adsorbent, respectively. It should be noted that the ^{3}H-labelled compounds were chromatographed only to demonstrate how the starting material can be separated from its ^{125}I-labelled derivative.

3. RESULTS AND DISCUSSION

Previous findings have been used in the present work, namely the fact that phenols, phenol derivatives and iodine-substituted phenol derivatives, such as organic solvents, e.g. methanol and ethanol tetrahydrofuran, are reversibly adsorbed on Sephadex LH-20 dextran gel [5, 6]. In an organic solvent/water binary eluent, water can be considered as an inert diluent which does not contribute to the eluent strength. Thus the eluent strength depends only on the concentration of the organic solvent (e.g. ethanol) according to

$$\log k = \log k_0 - n \log x \qquad (2)$$

where k stands for the distribution coefficient defined by Eq.(1) and x stands for the ethanol concentration expressed in mole fraction; k_0 and n are constants for a given solute. From Eq.(2) it turns out that the distribution coefficient of the starting material, the tracer and the labelled by-products can be adjusted to an optimum value by choosing the proper ethanol concentration of the eluent.

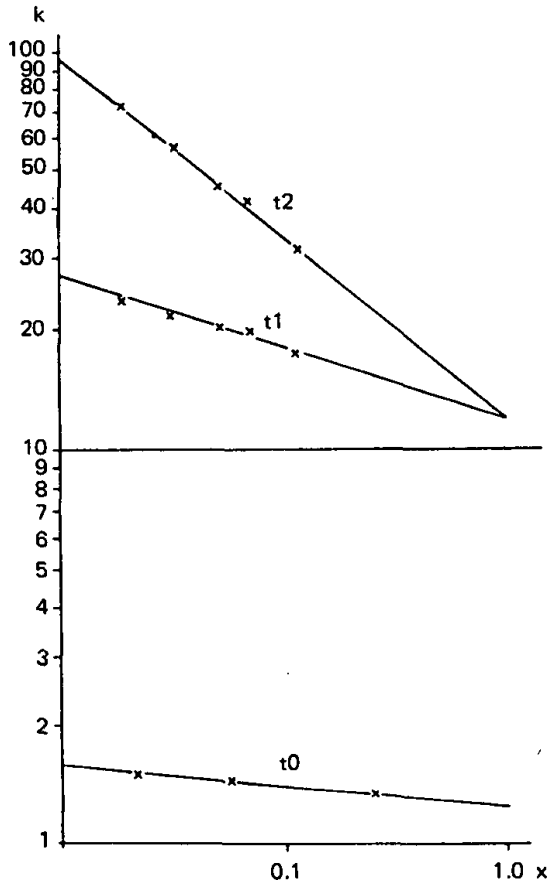

FIG.2. Plot of log k versus log x for L-tyrosine (t0), 3-iodo-L-tyrosine (t1) and 3,5-diiodotyrosine (t2) (eluent: water/ethanol, pH 4).

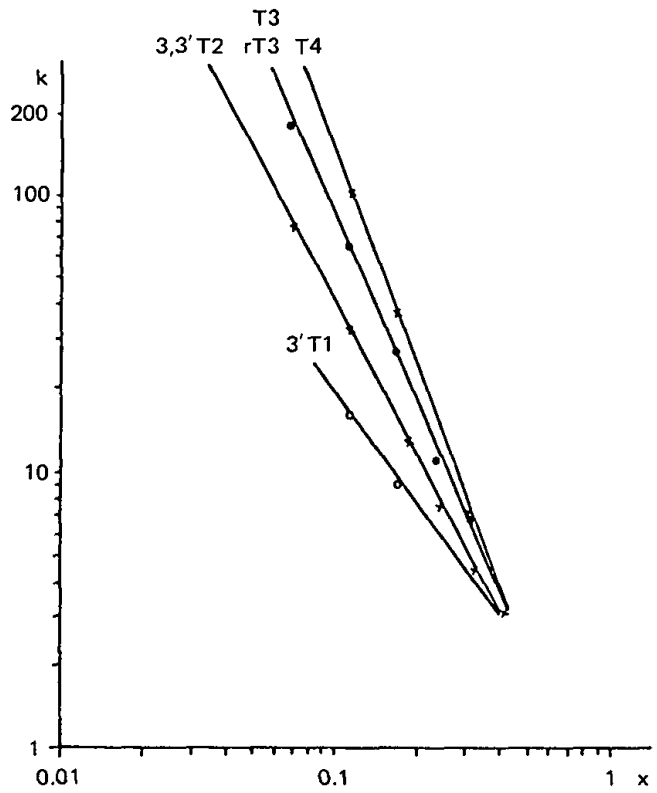

FIG.3. Plot of log k versus log x for iodothyronines (eluent: water/ethanol, pH 4).

3.1. Separation of iodotyrosines and iodothyronines

Figure 2 shows a plot of k versus x for t0, t1 and t2, on a log-log scale. The three plots clearly indicate that the introduction of one or two iodine atoms in the tyrosine molecule drastically increases the elution volume and also the distribution coefficient. Since the log k versus log x plots spread fanwise, the selectivity of the separation of t0-t1 or t1-t2 increases with decreasing ethanol concentration.

Figure 3 shows a plot of log k versus log x for iodothyronines. The plots are linear and the absolute value of the slope increases with increasing number of iodine substituents per molecule. The fanwise spread of the plots (as in Fig.2 for t0, t1 and t2) makes it possible to increase the selectivity of the separation of T1-T2-T3-T4 by lowering the ethanol concentration. T3 and rT3, which contain three iodine atoms per molecule, behave identically, which indicates that iodine substituents in the inner and outer (phenolic) ring contribute to the same extent to the adsorption affinity of iodothyronines.

The purification of ^{125}I-labelled iodotyrosines or iodothyronines is preferably started with a dilute water/ethanol eluent which elutes the free radioiodine, the starting material and the labelled by-products which contain less

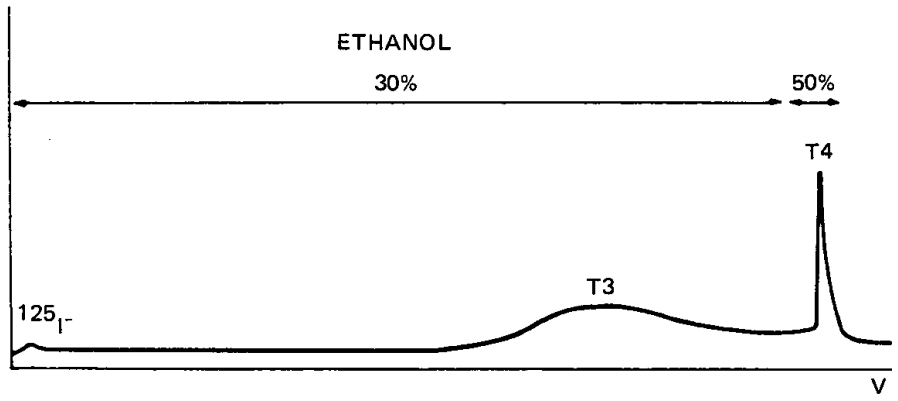

FIG.4. Elution pattern of the chloramine-T labelling mixture (starting material: T3, eluent: water/ethanol, pH 4).

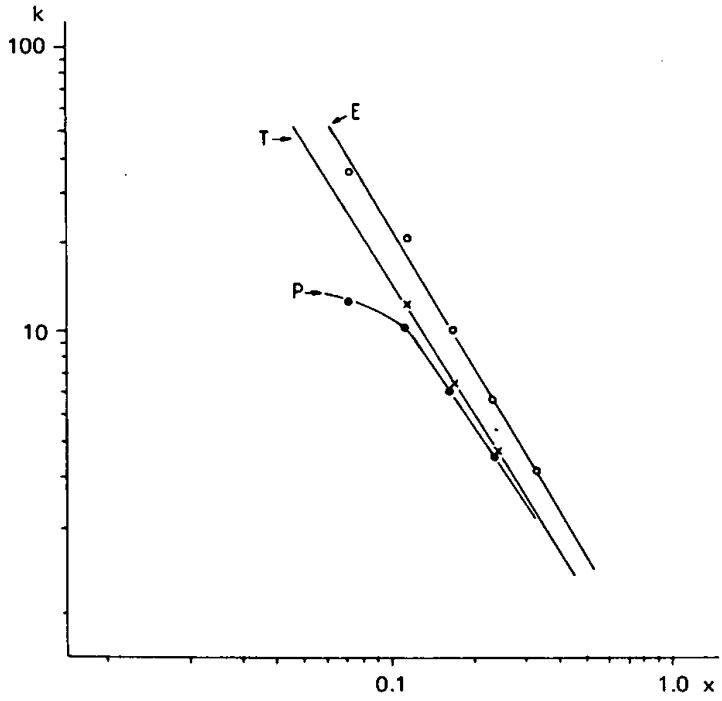

FIG.5. Plot of log k versus log x for ^{3}H-progesterone (●), ^{3}H-testosterone (x) and ^{3}H-estriol (○) (eluent: water/ethanol, pH 4).

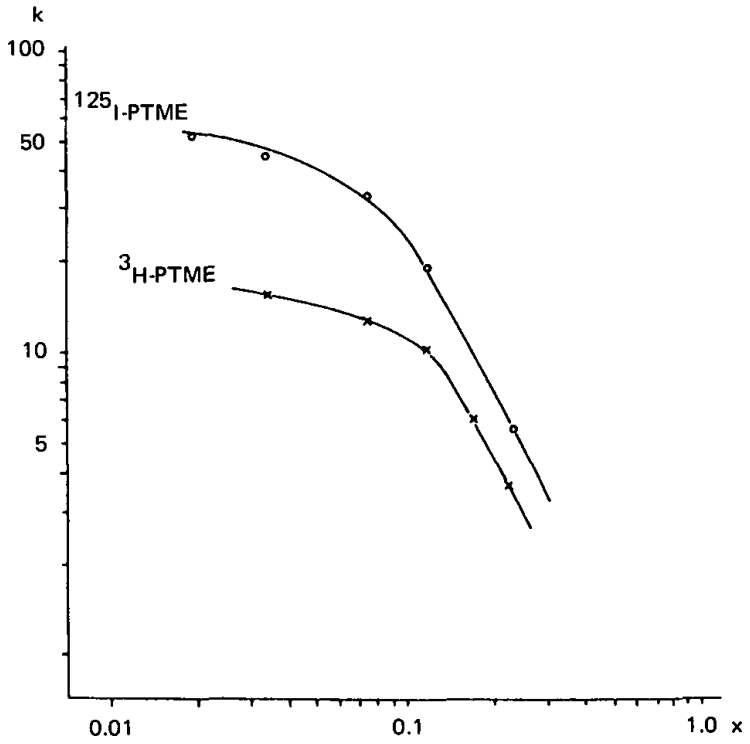

FIG. 6. Plot of log k versus log x for ^{3}H-PTME and ^{125}I-PTME (eluent: water/ethanol, pH 4).

iodine substituent per molecule than the tracer. The tracer can also be eluted with a more concentrated eluent. As illustrated in Fig.4 for ^{125}I-T4, with a stepwise elution the radioactive concentration of the tracer can be drastically increased. In addition, the increased ethanol concentration of the eluent increases also the shelf-life [7].

3.2. Separation of ^{125}I steroid/TME conjugates

It has been known for some time that steroids can be separated on a Sephadex LH-20 column using binary or ternary organic eluents [8]. However, no attempt has been made to establish a general relationship between the distribution coefficient and the eluent composition.

We have investigated the steroids and found a linear correlation of log k versus log x. The only exception is progesterone for which a deviation from linearity was observed for diluted eluents. The log k versus log x plots of steroids are presented in Fig.5.

A similar deviation from linearity was observed for progesterone-11-succinyl-TME and progesterone-11-succinyl-^{125}I-TME, as illustrated in Fig.6. The log k versus log x plots show that with decreasing ethanol concentration the selectivity of the separation increases considerably.

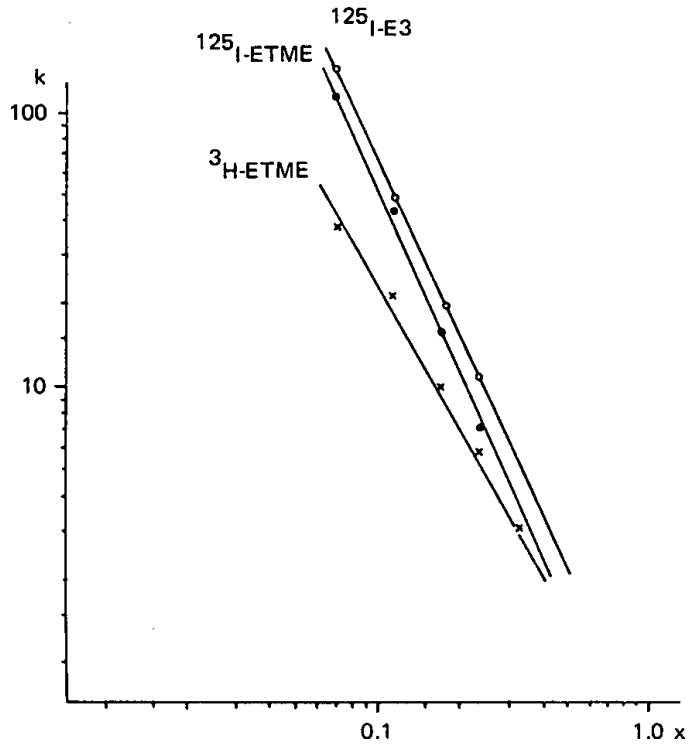

FIG.7. Plot of log k versus log x for ^{3}H-ETME, ^{125}I-ETME and ^{125}I-E3 (eluent: water/ethanol, pH 4).

For strongly adsorbed steroids like estriol, for its carboxymethyl/oxime TME derivative as well as for the ^{125}I-labelled derivative of the latter the log k versus log x plots are linear and parallel (Fig.7). The elution order is estriol, estriol-6-/O-carboxymethyl/oxime-TME and estriol-6-/O-carboxymethyl-^{125}I-TME. Surprisingly, the carboxymethyl oxime TME side-chain does not affect the retention of estriol, but the introduction of the ^{125}I atom into the TME residue at position 3 remarkably increases the distribution coefficient.

Estriol-like 17β-estradiol and estrone, which contain an aromatic A-ring, can be labelled directly with ^{125}I at positions 2 and/or 4; however, ring-labelled steroids usually show reduced or no binding ability to the antiserum [9]. Since the simultaneous labelling of estriol-6-/O-carboxymethyl/oxime-TME at positions 2 or 4 in the A-ring and at positions 3 or 5 in the TME cannot be precluded, the chromatographic behaviour of 2- or 4-iodo-estriol was also investigated (see Fig.7). The amount of ^{125}I in the labelling mixture was substoichiometric as compared with that of estriol and thus the formation of 2,4-diiodo-estriol should not be taken into account.

As expected, the A-ring-labelled estriol showed less than 1% binding to excess antiserum raised against estriol-6-/O-carboxymethyl/oxime-BSA, whereas the binding of estriol-6-/O-carboxymethyl/oxime-^{125}I-TME to the same antiserum surprisingly exceeded 90%.

Assuming that ^{125}I can be incorporated via aromatic electrophilic substitution into both the A-ring and the TME residue, it could be anticipated that the ^{125}I-labelled estriol-6-/O-carboxymethyl/oxime-TME tracer consists of a mixture of A-ring- and TME-labelled derivatives of which only the latter is bound to the antibody. Consequently, the maximum binding would be 50%, provided that the rates of the substitution reaction in the A-ring and in the phenolic group of TME do not differ significantly. The high binding of the ^{125}I-estriol-6-/O-carboxymethyl/oxime-TME to the antiserum indicates that only a TME-labelled derivative is formed. The fact that no A-ring-labelled derivative has been formed can be explained either by steric hindrance or by the low reaction rate.

4. CONCLUSIONS

It was found that the components of the chloramine-T labelling mixture, i.e. starting material, tracer, labelled by-products and free radioiodine, can be separated with a water/ethanol binary eluent and with Sephadex LH-20 dextran gel as adsorbent. The elution volume and the distribution coefficient can be adjusted to an optimum value by adjusting the ethanol concentration. Thus a tailor-made separation of the tracer can be achieved.

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