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Note

Adsorption chromatographic separation of ^{125}I-labelled cortisol-3-(O-carboxymethyl)oxime tyrosine methyl ester

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^{125}I can be introduced into cortisol-3-(O-carboxymethyl)oxime tyrosine methyl ester (CTME) via aromatic electrophilic substitution into the 3- and/or 5-position of the TME residue. When the ^{125}I in the labelling mixture is sub-stoichiometric relative to the CTME, only the formation of the monoiodo derivative needs to be taken into account. On the other hand, when CTME is applied in a 10–50-fold excess so as to suppress the formation of the diiodo derivative, the monoiodo-CTME, which is used as tracer in radioimmunoassay, has to be completely separated from the starting material. The inactive starting material, *i.e.*, CTME co-separated with the [^{125}I]CTME, drastically decreases the binding of the tracer to the antibody.

The separation of CTME and ^{125}I-labelled CTME can be performed by making use of the finding that iodine substituent(s) enhance the adsorption on Sephadex LH-20 dextran gel compared with the parent molecule[1–5].

Systematic investigations showed that the distribution coefficient, k, of radio iodine-labelled substances varies with the organic solvent concentration of the eluent according to

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

where X is the concentration of the organic solvent expressed as a molar fraction in the binary eluent and k_0 and n are constants for a given binary eluent and iodo compound.

EXPERIMENTAL

The labelling method, apparatus and adsorbent used have been described previously[1–4]. CTME was labelled with ^{125}I by the use of the chloramine-T method. To 25–50 μg (50–100 nmole) of CTME (mol.wt. 612) dissolved in 25–50 μl of ethanol, 100 μl of phosphate buffer (pH 7.6) and 1–2 mCi (0.5–1.0 nmole) of carrier-free ^{125}I were added, followed by 50 μl of solution containing 200–300 μg of chloramine-T. The labelling reaction was quenched after 30–60 sec with 700 μg of sodium metabisulpite in 100 μl. In the course of the chloramine-T labelling ^{125}I is introduced via aromatic electrophilic substitution in the 3- and/or 5-position of the tyrosine methyl ester residue (Fig. 1).

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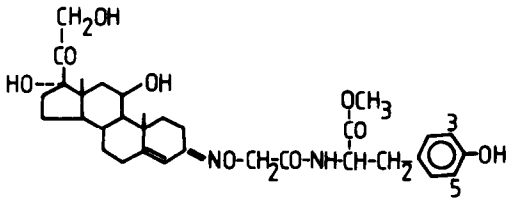

Fig. 1. Cortisol-3-(O-carboxymethyl)oxime tyrosine methyl ester.

Sephadex LH-20 dextran gel was swollen in distilled water prior to being packed in the column (130 × 10 mm I.D.). The height of the packing was 100 mm. In order to check the separation of the starting material (CTME) in the labelling procedure from the ^{125}I-labelled CTME and free radioiodine, tritium-labelled cortisol (Amersham TRK-407) was also chromatographed separately from the chloramine-T labelling mixture.

The sample (0.1–0.2 ml) was placed on the top of the column and allowed to soak in; 10–20 min later, *i.e.*, when adsorption equilibrium had been attained, elution was performed with ethanol–water.

When [^{3}H]cortisol was chromatographed, the effluent was collected with a fraction collector and its radioactivity was determined by liquid scintillation counting. With the ^{125}I labelling mixture 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. A peristaltic pump, flow-rate 22–24 ml/h, delivered the eluent.

The distribution coefficient was calculated according to

$$k = \frac{V_e - V_0}{W} \tag{2}$$

where V_e, V_0 and W are the elution volume, the dead volume and the weight of the adsorbent, respectively.

RESULTS

Fig. 2 shows the distribution coefficient of [^{3}H]cortisol and that of [^{125}I]CTME as a function of the ethanol concentration, on a log–log scale. Log k is linearly dependent on log X in accordance with eqn. 1:

$$\log k = 0.08 - 0.41 \log X \text{ for } [^3\text{H}]\text{cortisol} \tag{3}$$

$$\log k = 0.02 - 1.36 \log X \text{ for } [^{125}\text{I}]\text{CTME} \tag{4}$$

As these two relationships diverge, the selectivity of the separation, defined as the ratio of the distribution coefficients ($\alpha = k_2/k_1$), increases with decreasing ethanol concentration.

As can be seen from Fig. 3, an increase in pH does not affect the distribution coefficient up to pH 8, but a further increase in pH results in a sudden drop. This can be attributed to the dissociation of the phenolic hydroxyl group of the TME residue, which has the same effect in iodothyronines[1] and iodotyrosines[5]. On the

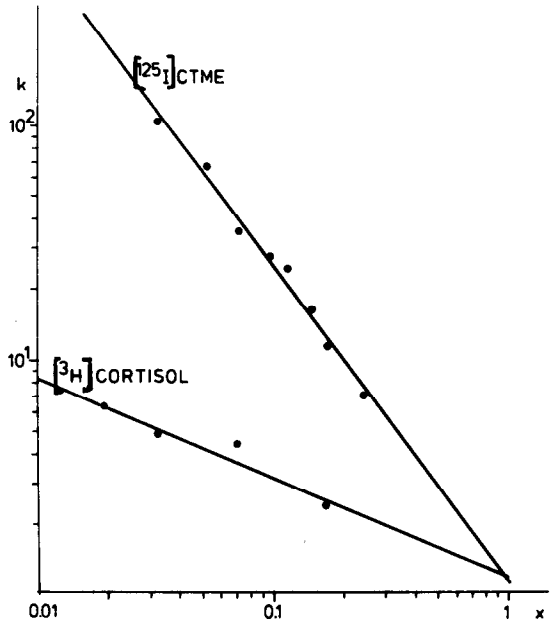

Fig. 2. Log k *vs.* log X plots for [^{3}H]cortisol and [^{125}I]CTME (pH 4; 25°C).

other hand, pH has no effect on the distribution coefficient of the tritium-labelled cortisol between pH 1 and 13.

From Fig. 3, it can be concluded that the adsorption affinity of [^{125}I]CTME can be attributed partly to the steroid skeleton and partly to the TME residue.

The finding that the k value of [^{125}I]CTME in the pH range 1–8 at constant ethanol concentration (20%) is considerably higher than that of [^{3}H]cortisol indicates that mainly the iodo-TME residue is responsible for the adsorption of the [^{125}I]CTME molecule. The decrease of k for [^{125}I]CTME with increasing pH commence around pH 8 and is completed at pH 12. At higher pH the k values of [^{3}H]cortisol and [^{125}I]CTME become equal, from which it can be concluded that the dissociation of the phenolic hydroxy group cancels the adsorption affinity of the

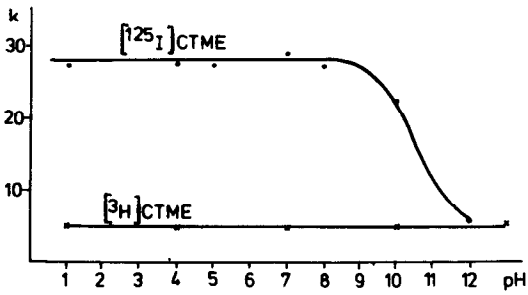

Fig. 3. Effect of pH on the distribution coefficients of [^{3}H]cortisol and [^{125}I]CTME (eluent 20% ethanol; 25°C).

TME residue and thus at pH 12 k reflects the adsorption affinity of the steroid skeleton towards the Sephadex LH-20 gel only.

The adsorption molecular model proposed by Soczewinski and co-workers for polar adsorbents such as silica[6,7] is analogous to the model applied by us for non-polar Sephadex LH-20 adsorbent and iodothyronine solutes[1]. The greatest difference between the two models is that in the former instance it is the polar and in the latter instance the non-polar component of the binary eluent that is the active component and the other one can be considered to be an inactive diluent.

The simplified adsorption model[1,6,7] neglects any interaction between solvent and solute molecules both in the liquid phase and in the adsorbed state, and takes into account only the competitive adsorption of solvent and solute. Assuming that a given chromatographic system meets all the requirements of this model, it can be shown that the slope of the log k *vs.* log X plot is equal to the average number of solvent molecules that displace one solute molecule from the surface of the adsorbent[1,6,7].

A slope greater than unity can be expected in the case of multi-point adsorption of the solute or even in the case of single-point adsorption, provided that more than one solvent molecule can displace one solute molecule from the surface.

Comparison of the log k *vs.* log X plots for [^{3}H]cortisol and [^{125}I]CTME reveals that both the steroid skeleton and the TME residue are adsorbed on the gel surface, *i.e.*, multi-point adsorption of [^{125}I]CTME occurs. The introduction of the iodo-TME side-chain results in an increase in n in eqn. 1 from 0.41 to 1.36.

When [^{125}I]CTME is used as a tracer in radioimmunoassay, it is recommended that free radioiodine and unlabelled CTME be eluted with 30% ethanol followed by 40–50% ethanol, which considerably increases the radiochemical concentration of [^{125}I]CTME.

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