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Note

Adsorption chromatographic separation of ^{125}I-labelled estriol and estriol-6-(O-carboxymethyl)oxime tyrosine methyl ester

GÉZA TÓTH
Institute of Isotopes of the Hungarian Academy of Sciences, P.O. Box 77, H-1525 Budapest (Hungary)
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Steroid molecules labelled with ^{125}I are being increasingly used instead of tritium-labelled molecules as tracer in the radioimmunoassay. Although radioiodine can be incorporated via electrophilic substitution into steroids with an aromatic A-ring in the 2- and/or 4-positions[1,2], the iodination of the steroid itself in the A-ring is considered to result in the complete loss of tracer affinity to the antibody[3,4]. Therefore, radioiodine is usually incorporated via electrophilic substitution into a side-chain [tyrosine methyl ester (TME), histamine, etc.] coupled to the steroid skeleton. In steroids with an aromatic A-ring (*e.g.*, estriol and 17-β-estradiol), electrophilic substitution takes place simultaneously in the 2- and/or 4-position of the A-ring and in the 3- and/or 5-position of the TME side-chain.

In order to avoid the formation of the A-ring-labelled 17-β-estradiol-6-(O-carboxymethyl)oxime histamine. Nars and Hunter[3] conjugated the already iodinated [^{125}I]histamine to the 6-(O-carboxymethyl)oxime derivative of the 17-β-estradiol, extracted the tri-*n*-butylamine and isobutyl chloroformate activators with toluene and separated the radioiodinated 17-β-estradiol-6-(O-carboxymethyl)oxime by thin-layer chromatography (TLC). Finally, the ^{125}I-labelled 17-β-estradiol-6-(O-carboxymethyl)oxime histamine was eluted from the silica powder scraped from the plate[3].

The aim of this paper is to show that estriol-6-(O-carboxymethyl)oxime tyrosine methyl ester (ETME) labelled with ^{125}I in the tyrosine methyl ester (TME) side-chain can be separated from the inactive starting material and from an unidentified labelled by-product using Sephadex LH-20 dextran gel as the adsorbent and ethanol–water as the eluent.

When ETME (see Fig. 1) is labelled with ^{125}I by the use of the chloramine-T method, the simultaneous formation of labelled 2-, 3′- and 4-iodo-ETME and 2,3′-,

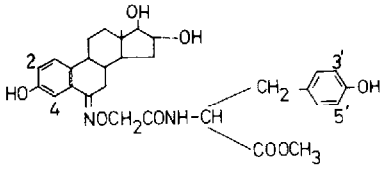

Fig. 1. Estriol-6-(O-carboxymethyl)oxime tyrosine methyl ester. The figures indicate the positions into which radioiodine can be introduced via electrophilic substitution.

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2,4- and 3',5'-diido-ETME has to be taken into account. Nevertheless when the amount of ^{125}I used in the chloramine-T labelling procedure is sub-stoichiometric compared with that for ETME, the formation of the diiodo derivatives can be precluded. Of the monoiodo-ETME derivatives, only 3'-iodo-ETME is expected to be bound to the antiserum raised against the same derivative, *i.e.*, estriol-6-(O-carboxymethyl)oxime bovine serum albumin (BSA).

EXPERIMENTAL

The apparatus and adsorbent used have been described previously[5–8]. ETME and estriol were labelled with ^{125}I by the use of a slightly modified version of the chloramine-T method which will be published later. To 10–20 μg (20–40 nmole) of ETME (mol.wt. = 552) or estriol dissolved in aqueous ethanol 100 μl of phosphate buffer (pH 7.6) were added, then 1–2 mCi (37–74 MBq) of carrier-free ^{125}I (0.5–1.0 nmole) followed by the addition of 20–50 μl of an aqueous solution containing 100–200 μg of chloramine-T. The labelling reaction was quenched after 30–60 sec with 50 μl of an aqueous solution containing 350 μg of sodium metabisulphite.

Sephadex LH-20 dextran gel was swollen in citrate buffer (pH 4) 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 ETME from the ^{125}I-labelled ETME, tritium-labelled ETME was also chromatographed separately from the chloramine-T labelling mixture.

In all instances the sample (0.1–0.2 ml) from the chloramine-T labelling procedure was placed on the top of the column and allowed to soak in it. After 10–20 min, *i.e.*, when adsorption equilibrium had been attained, the elution was performed with aqueous ethanol, the pH of which was adjusted to 4 so as to supress the dissociation of the phenolic hydroxyl groups. The effluent was passed over a NaI(Tl) scintillation crystal and the count rate monitored by a ratemeter and registered by an x–y plotter. A peristaltic pump, flow-rate 22–24 ml/h, delivered the eluent.

When tritium-labelled ETME was chromatographed the effluent was collected with a fraction collector and its radioactivity was determined by liquid scintillation counting.

The distribution coefficient was calculated according to

$$K = \frac{V_e - V_0}{W} \tag{1}$$

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

RESULTS AND DISCUSSION

The elution pattern obtained when tritium-labelled ETME was chromatographed is shown in Fig. 2. Omitting the elution peak of the free radioiodine, the elution pattern recorded when the chloramine-T labelling mixture of ETME was chromatographed is shown in Fig. 3. Of the two elution peaks the first was assigned to an unidentified labelled by-product. The second peak is attributed on the basis of the immunoreactivity (see later) to 3'-iodo-ETME.

Fig. 2. Elution pattern of [^{3}H]ETME. Eluent: 30% aqueous ethanol (pH 4).

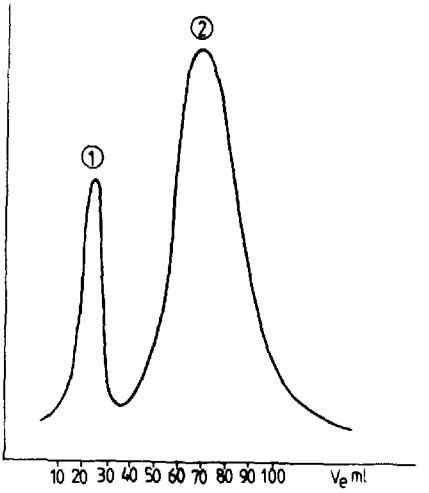


Fig. 3. Elution pattern of the chloramine-T labelling mixture for ETME. Eluent: 30% aqueous ethanol (pH 4). Peak 1 = unidentified labelled compound; peak 2 = [3'-^{125}I]ETME.

The elution volume of the labelled by-product and that of the 3'-iodo-ETME decreases monotonously with increasing ethanol concentration of the eluent. This is illustrated in Fig. 4, which shows the elution patterns obtained at different ethanol concentrations. It can be seen from these elution curves that with a 40% ethanol concentration the separation is unsatisfactory whereas with 50% ethanol no separation of the two labelled products can be achieved at all. With 30% ethanol the difference in the elution volumes of ETME (26 ml) and 3'-iodo-ETME (78 ml) is large enough to achieve complete separation of the starting material and the 3'-iodo-ETME (see Figs. 2 and 3).

Estriol was also labelled with ^{125}I using the same procedure as in the case of ETME. The elution pattern obtained when the labelling mixture of estriol was chromatographed is shown in Fig. 5. As the formation of 2,4-diiodoestriol can be precluded owing to the large excess of the starting material compared with the amount of ^{125}I (*i.e.*, 20–40 nmole to 0.5–1.0 nmole) the elution peak seen in Fig. 5 can only be attributed to 2- or 4-iodoestriol.

Immunoreactivity of the ^{125}I-labelled estriol and ETME

In order to check the immunoreactivity of [^{125}I]estriol and [^{125}I]ETME, the labelled compounds corresponding to the elution peaks shown in Figs. 3 and 5 were incubated with antiserum raised against estriol-6-(O-carboxymethyl)oxime bo-

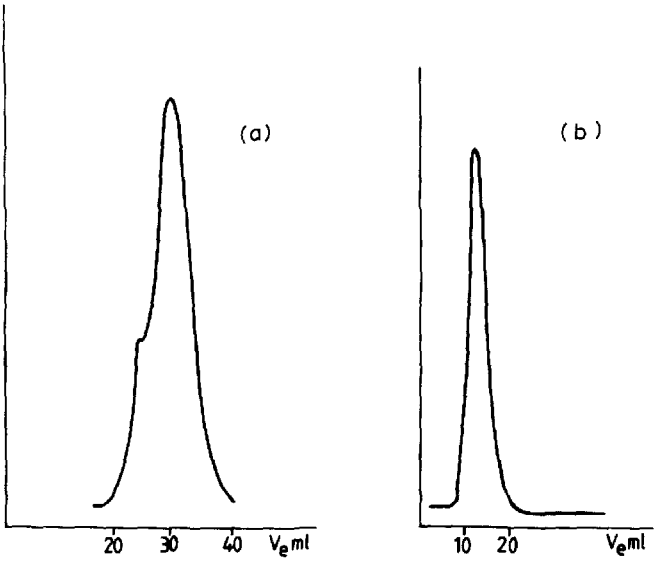


Fig. 4. Effect of ethanol concentration on the separation of the chloramine-T labelling mixture for ETME. Eluent: (a) 40% aqueous ethanol (pH 4); (b) 50% aqueous ethanol (pH 4).

vine serum albumin conjugate. As the antiserum does not distinguish between estriol and [^{125}I]ETME (labelled in the tyrosine residue) on the one hand, and does not bind A-ring-labelled estriol or ETME on the other, it was expected that only 3'-iodo-ETME (*i.e.*, the second elution peak in Fig. 3) would exhibit immunoreactivity. This is clearly supported by the data in Table I.

Effect of the organic solvent concentration on separation

The distribution coefficient (K) *versus* ethanol concentration in the eluent relationship is

$$\log K = \log K_0 - n \log x \tag{2}$$

where x is the ethanol concentration in the eluent expressed as a molar fraction, K_0 is the distribution coefficient extrapolated to $x = 1$ and n is a constant. Inserting actual K_0 and n values, K can be expressed as

$$\log K = -0.69 - 2.4 \log x \text{ for 2- or 4-iodoestriol} \tag{3}$$

$$\log K = -0.52 - 2.3 \log x \text{ for 3'-iodo-ETME} \tag{4}$$

TABLE I

BINDING OF ^{125}I-LABELLED ESTRIOL AND ETME DERIVATIVES

^{125}I-labelled derivative	*Binding (%)*
2- or 4-iodoestriol	<1
3'-Iodo-ETME	60–70

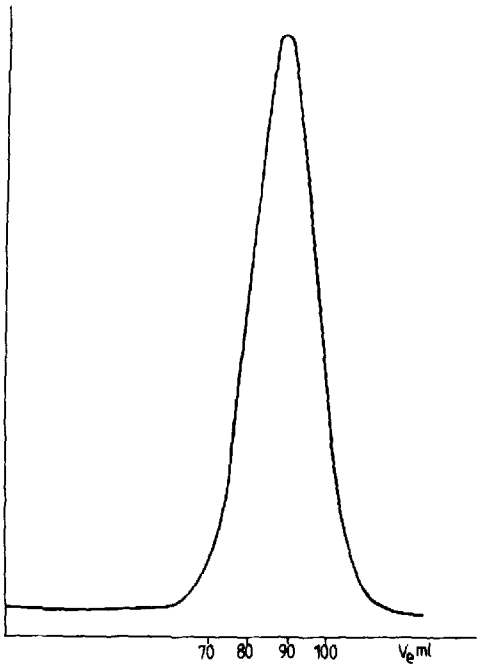


Fig. 5. Elution pattern of the chloramine-T labelling mixture for estriol. Eluent: 30% aqueous ethanol (pH 4).

Eqn. 2 also holds for ^{125}I-labelled iodothyronines[5], prostaglandins[6], testosterone[7] and progesterone[8].

The log K *versus* log x relationship for 2- and 4-iodoestriol and 3'-iodo-ETME is shown in Fig. 6.

CONCLUSIONS

2 and/or 4-iodoestriol and 3'-iodo-ETME are reversibly adsorbed on Sephadex LH-20 dextran gel and their selective elution can be performed by the use of water–ethanol as the eluent. The distribution coefficient defined by eqn. 1 varies with the organic solvent concentration of the solvent according to eqn. 2.

According to expectation, chloramine-T labelling of estriol results in the formation of 2- or 4-iodoestriol only if the amount of ^{125}I is sub-stoichiometric compared with that of the estriol. As the Sephadex LH-20 dextran gel does not distinguish between 2- and 4-iodoestriol, only a single elution peak appears when the labelled mixture of estriol is chromatographed. With ETME the electrophilic substitution of ^{125}I may take place either in the A-ring or in the tyrosine residue. Thus two labelled ETME derivatives can be expected to be formed in the chloramine-T labelling procedure.

Actually, apart from an unidentified labelled by-product there is only a single labelled product formed, which because of its immunoreactivity is attributed to 3'-iodo-ETME. The lack of formation of A-ring-labelled ETME might be due to the steric hindrance caused by the 6-(O-carboxymethyl)oxime TME side-chain or by the

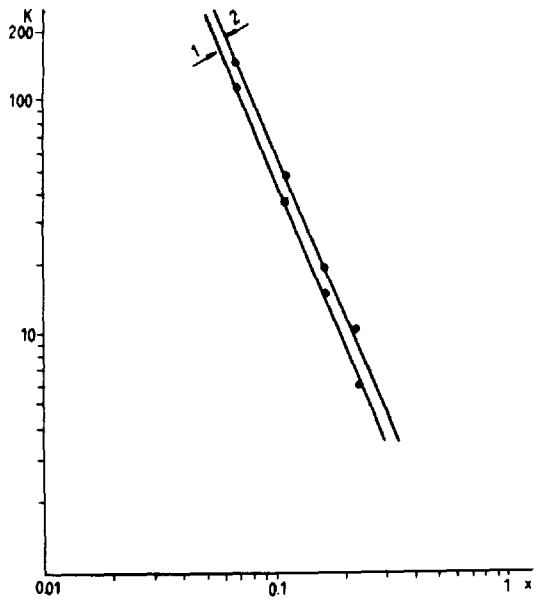
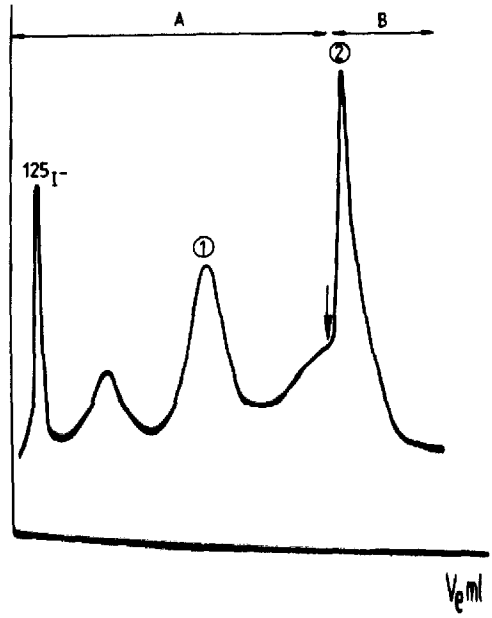


Fig. 6. Distribution coefficient *versus* ethanol concentration. 1 = 2- or 4-iodoestriol; 2 = 3′-iodo-ETME.

lower rate of substitution of iodine into the A-ring compared with that into the TME side-chain.

When the chromatography is aimed at the production of high specific activity and radiochemically stable ETME labelled in the tyrosine residue, it is recommended that free radioiodine and the ^{125}I-labelled by-product is eluted with 30% aqueous ethanol followed by elution of the immunoreactive [3′-^{125}I]ETME with 50% aqueous ethanol. The elution pattern thus obtained is shown in Fig. 7.

The [3′-^{125}I]ETME thus obtained can be used as a tracer in the radioimmunoassay of estriol at least for 3 month. The long shelf-life is attributed to the low



dielectric constant (relative permittivity) of the solvent, which substantially increases the radiochemical stability[9].

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