CHROM. 5385

Thin-layer chromatography and elution of picogram amounts of estradiol

The recent development in protein-binding methods (also called saturation analysis[1]) has made it possible to determine picogram amounts of estradiol (E_2, estra-1,3,5(10)-triene-3,17β-diol) in biological samples utilizing anti-estradiol antibodies[2–4] or receptor proteins from the uterus of various species[5,6]. Although these proteins are highly specific, at least one chromatographic step is usually required to separate E_2 from a few crossreacting estrogens and from some unknown material which interferes with the endpoint determination. This study was initiated during the development of a hapten-radioimmunoassay[7]. We realized that it is not possible to conclude that a chromatographic system and elution procedure which will permit quantitative recoveries in the microgram range, will also be reliable when applied to nano- or picogram amounts of that particular compounds. Therefore conditions were developed which allow artifact free thin-layer chromatography (TLC) and elution of picogram amounts of E_2 and yield blank values in the endpoint determination by a hapten-radioimmunoassay system below 10 pg.

Materials

All reagents were analytical grade and purchased from Merck AG Darmstadt, unless otherwise stated. Ethanol, 2-mercaptoethanol (puriss. Serva, Heidelberg), phenol and naphthalene were used without further purification. Benzene and ethyl acetate were distilled through a Vigreux column. Methanol was redistilled after treatment with 2,4-dinitrophenylhydrazine[8]. Dichloromethane was treated with 0.1 vol. conc. sulfuric acid for a few days, washed once with 0.1 vol. 2 *N* NaOH, then three times with 0.1 vol. demineralized water, dried over anhydrous sodium sulfate and distilled. The purity of estradiol-17β was checked by TLC using the solvent systems benzene–ethyl acetate (75:25) and benzene–ethanol (90:10). Estradiol-17β-6,7-^{3}H (40 Ci/mmole) and estradiol 17β-2,4,6,7-^{3}H (107 Ci/mmole) were obtained from New England Nuclear Corp. A purity greater than 95 % was established. For this purpose 3 μg unlabeled and 100,000 d.p.m. labeled estradiol were spotted on TLC plates and developed in the systems already mentioned. After scanning (Dünnschicht-Scanner II, Berthold/Frieske GmbH, Karlsruhe-Durlach), the estradiol peak and the remaining radioactivity were eluted separately with methanol into counting vials, and after evaporation and addition of a toluene based scintillator the radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer (Model 3375).

The thin-layer plates, 20 × 20 cm, precoated with Silica Gel F254 (Merck AG, Darmstadt, catalog number 5715/0025), were purified by ascending chromatography in methanol three times, air dried for 1/2 h and stored in a tight case specially designed for this purpose (Desaga, Heidelberg). All glassware used was cleaned with a detergent, rinsed with tap water and demineralized water. Nitrogen, purified, was obtained from LINDE AG, München-Lohhof, methane from the laborabory Prof. Dr. BERTHOLD, Wildbad.

Methods

Standard procedure. 100,000 d.p.m. of E_2-6,7-^{3}H (total mass 306 pg) dissolved in 1 μl benzene containing 10 % ethanol were spotted four times on the starting line of the TLC plate at a distance of 4 cm from each other. On each side 5 μg E_2 were placed as a marker. One or two plates were placed in a tank lined with filter paper and saturated for at least 2 h with the solvent system benzene–ethyl acetate (75:25). After the solvent front had reached the 15 cm line the plates were taken out for 3–5 min and the chromatography was then repeated as before. The first chromatography is called Ia, the second one Ib. After localisation of the marker spots by UV absorbtion the corresponding areas of the labeled E_2 (2 × 3 cm) were scraped off with an adapted razor blade and sucked into a Pasteur pipette plugged with glass wool. The E_2 was eluted three times with 1 ml of 30 % methanol in dichloromethane into small culture tubes. After evaporation in nitrogen at 40° the residues were spotted on TLC plates using about 100 μl 10 % methanol in dichloromethane three times. Again the chromatogram was developed twice in the system benzene–ethyl acetate (75:25) (chromatography IIa and IIb).

Variations of the standard procedure. (1) Instead of 306 pg of labeled E_2 (corresponding to 100,000 d.p.m. E_2-6,7-^{3}H, 40 Ci/mmole) 81 and 40.5 pg (corresponding to 70,600 and 35,300 d.p.m. of E_2-2,4,6,7-^{3}H, 107 Ci/mmole) were used.

(2) 100,000 d.p.m. E_2-6,7-^{3}H (40 Ci/mmole) were mixed with various amounts of unlabeled E_2 (3 μg, 500 ng, 50 ng, 5 ng, 0.5 ng), chromatographed, eluted, and rechromatographed as described.

(3) The interval between the end of chromatography Ib and the start of chromatography IIa was varied. The plates, protected only from sunlight, were exposed to the air in the laboratory before elution was started.

(4) The benzene–ethyl acetate system was modified by adding: (a) 100 mg phenol; (b) 100 μl 2-mercaptoethanol; or (c) 100 mg naphthalene to 100 ml of the solvent mixture.

(5) Instead of the benzene–ethyl acetate system benzene–ethanol (90:10) was used.

(6) TLC plates spotted only with the marker E_2 were chromatographed in the benzene–ethyl acetate system containing either phenol or 2-mercaptoethanol. At the height of the marker E_2 areas (2 × 3 cm) were scraped off and eluted as described. The residues after evaporation were tested as blank values in a hapten-radioimmunoassay system.

Results

Fig. 1 (lanes 1 and 2) demonstrates the extensive artifact formation in the case that only 300 pg E_2-6,7-^{3}H (40 Ci/mmole) are chromatographed, eluted, and rechromatographed as described under *Standard procedure*. The E_2 peak is incompletely separated from the degradation products, some of which are less polar and some more polar than E_2 in that particular chromatographic system. No attempt has been made to identify these products. By adding 3 μg unlabeled E_2 to 300 pg labeled E_2 artifact formation becomes negligible (lanes 3 and 4). If instead of the benzene–ethyl acetate system benzene–ethanol (90:10) was used the degree of artifact formation was

essentially the same, but the chromatographic behavior of the degradation products was slightly different.

The degree of artifact formation as a function of the total mass of E_2 is shown in Fig. 2 (lane 1: 300 pg labeled E_2 + 0.5 ng unlabeled E_2; lane 2: + 5 ng E_2; lane 3: + 50 ng E_2; lane 4: + 500 ng E_2. It can be seen that 500 ng E_2 are required to prevent artifact formation.

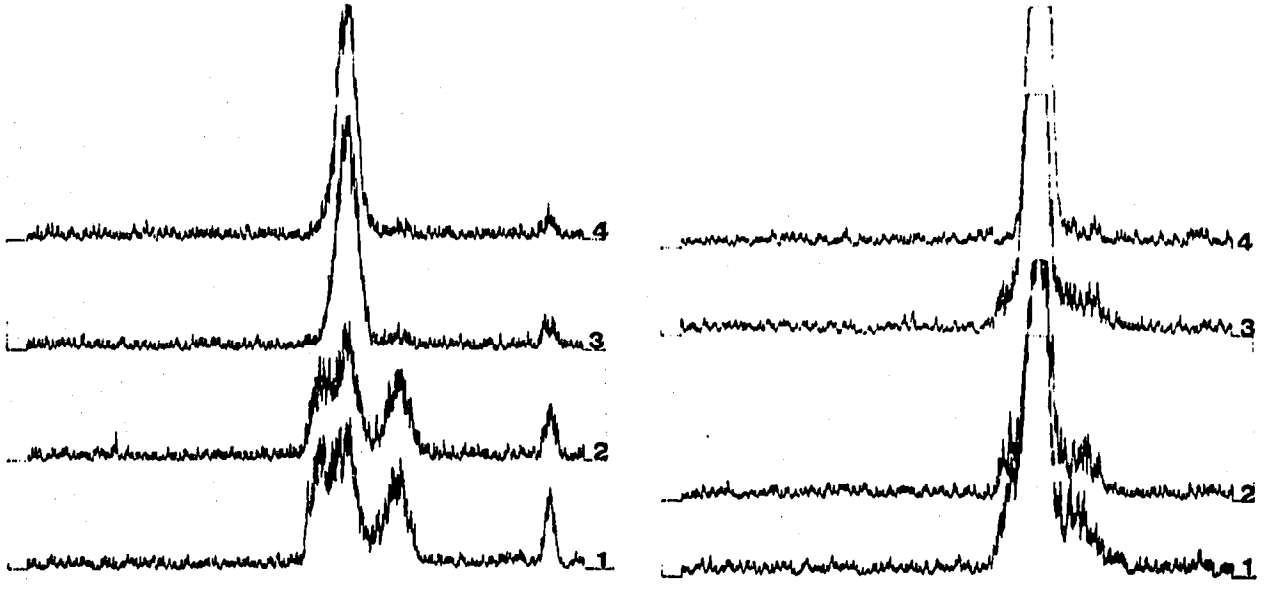

Fig. 1. Radiochromatogram after TLC, elution, and a second TLC as described (*Standard procedure*). Lanes 1 and 2, 300 pg tritiated E_2; lanes 3 and 4, 300 pg tritiated E_2 + 3 μg unlabeled E_2.

Fig. 2. Chromatography, elution, and rechromatography of 300 pg tritiated E_2 + 0.5 ng unlabeled E_2 (lane 1), + 5 ng E_2 (lane 2), + 50 ng E_2 (lane 3), + 500 ng E_2 (lane 4).

Fig. 3 illustrates that the longer E_2 is adsorbed onto the silica gel (exposed to the air at room temperature) the greater is the extent of artifact formation. Each time 300 pg labeled E_2 were used according to the standard procedure. In case of lane 1 working up the sample between the end of chromatography Ib and the start of chromatography IIa was performed as rapidly as possible and took about 10 min. For lane 2 this interval was 1 h; for lane 3, 5 h; for lane 4, 18 h.

Fig. 4 demonstrates that artifact formation on the TLC plates is prevented by adding a small amount of a mild reducing agent to the chromatographic system. Lane 1 is the chromatogram of the control experiment performed simultaneously. The time between the end of chromatography Ib and the start of chromatography IIa was 1 h, all the other details were identical with the standard procedure. Lane 2: This experiment differs from the control experiment only in the fact that 100 mg phenol were added to 100 ml of the solvent system. Lane 3: Phenol was replaced by 100 μl 2-mercaptoethanol. Lane 4: Phenol was replaced by 100 mg naphthalene. The last experiment confirms that the protective effect of phenol and mercaptoethanol is due to their reducing properties.

The experiments summarized in Fig. 4 were repeated with 80 and 40 pg E_2-2,4,6,7-^{3}H (107 Ci/mmole). The chromatograms are essentially the same as those

shown in Fig. 4, indicating that amounts as small as 40 pg E_2 can be chromatographed without artifact formation.

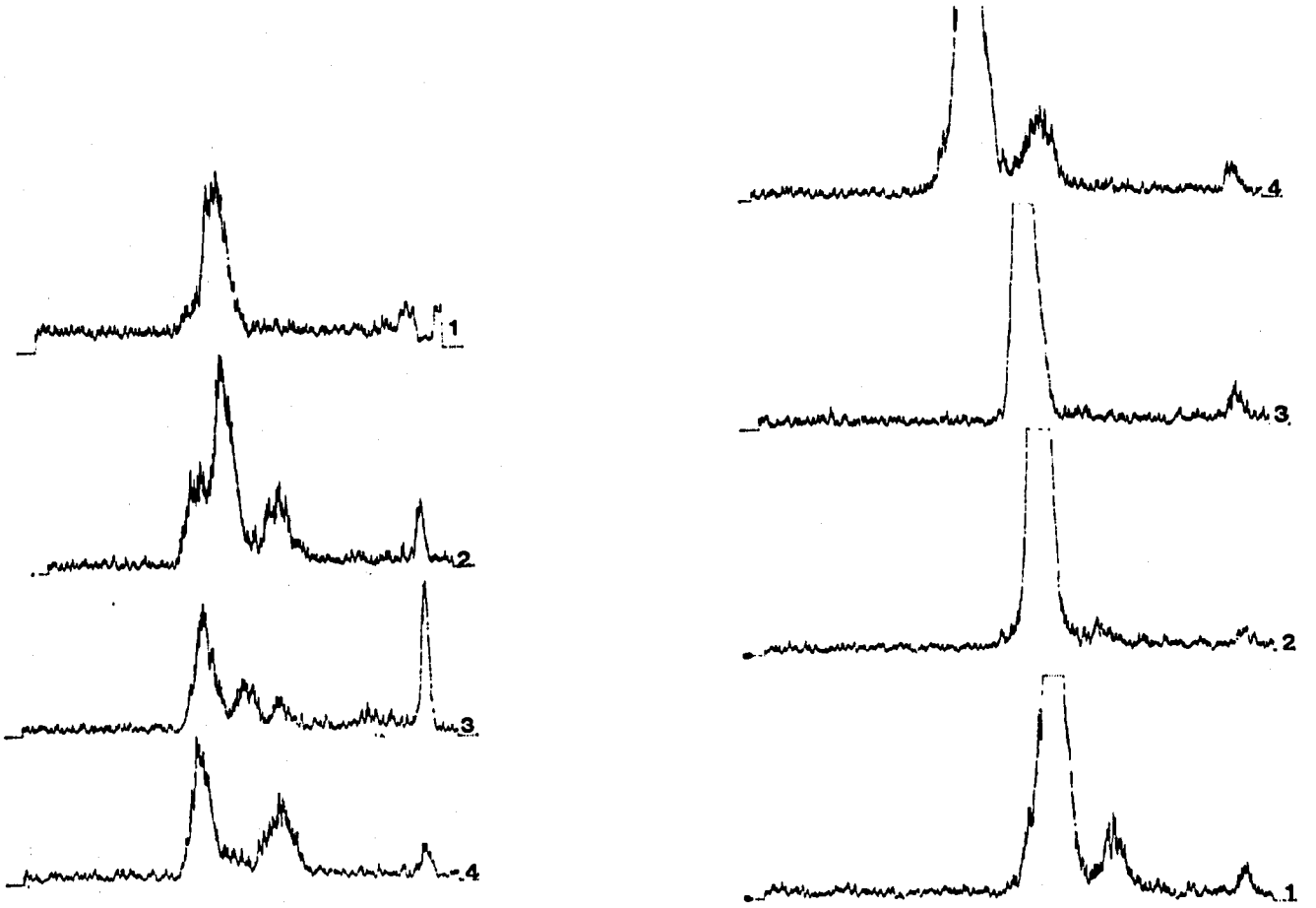

Fig. 3. Chromatography, elution, and rechromatography of 300 pg tritiated E_2. The interval between the end of chromatography Ib and the start of chromatography IIa was 10 min (lane 1); 1 h (lane 2); 5 h (lane 3); 18 h (lane 4).

Fig. 4. Lane 1: control chromatogram as in Fig. 1, lanes 1 and 2. Lane 2: the benzene–ethyl acetate system was modified by adding 100 mg phenol per 100 ml solvent mixture. Lane 3: phenol is replaced by 100 μl 2-mercaptoethanol. Lane 4: phenol is replaced by 100 mg naphthalene.

The blank values of TLC plates after development in a system containing either phenol or mercaptoethanol were tested in a hapten-radioimmunoassay system (see *variation of the standard procedure* (6)) and were found to be constantly below 10 pg.

Discussion

The experiments reported demonstrate that under the specified conditions amounts as small as 40 pg tritiated E_2 survive chromatography on silica gel thin-layer plates, elution, and evaporation. It is assumed that the unlabeled compound is also stable under these conditions. The artifact formation depended on the amount of E_2 per surface area as well as on the duration of the adsorbtion of E_2 on the silica gel. Since the presence of reducing agents throughout the whole procedure could prevent the formation of artifacts, they were obviously caused by oxygen. There is no cogent reason to conclude that atmospheric contaminants are responsible for

this kind of artifact as has been claimed[9]. Since impurities in the solvents are known to cause degradation of various steroids during evaporation[10,11], especially in the presence of silica gel particles[12–15], we only used solvents of high purity.

We did not evaluate to what extent phenol or mercaptoethanol are removed after evaporation in nitrogen at 40°, since the blank values in the endpoint determination by a hapten–radioimmunoassay system were below 10 pg. If another kind of endpoint determination is to be used, the procedure has to be reconsidered.

It is possible that other compounds could also be protected from degradation during TLC if the principle involved in our procedure is utilized for this purpose.

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