Journal of Chromatography, 89 (1974) 374–379

CHROM. 7174

Note

Thin-layer chromatography of oestrogens, their derivatives and cholesterol

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(First received March 9th, 1973; revised manuscript received October 16th, 1973)

The use of thin-layer chromatography (TLC) in the separation of oestrone, 17β-oestradiol and cholesterol was investigated. TLC was also used to isolate derivatives suitable for gas–liquid chromatography from their parent oestrogens. Separations of about 3 cm (0.2 R_F units) were required since the low levels of oestrogens present in plasma samples made chromatogram evaluation by radioactivity scanning necessary —a spot-centre separation of 3 cm being the limit for the complete resolution of two peaks using the Tracerlab 2π radiochromatogram scanner. The elution of oestrogens and their monochloroacetate derivatives from silica gel was also studied.

Reports on the separation of oestrone from 17β-oestradiol by TLC[1–6] and from other steroids[7–9] have been concerned with at least six steroids. An exception is the use of Florisil thin layers by Nienstedt[10] to remove cholesterol with other lipids from hormonal steroids. TLC of oestrogen derivatives has been described[2,11–17] and the derivatives used here were readily separated from the oestrogens.

Various methods for elution of substances from adsorbent layers have been published and several solvent systems used[18–24]. Elution in the form of column chromatography (Attal *et al.*[25], as modified by Masaracchia and Gawienowski[26]) appeared simple, efficient and rapid, but it is reported[27,28] that highly polar solvents cause destruction of steroids during elution.

EXPERIMENTAL

Silica gel GF_{254} (nach Stahl; E. Merck, Darmstadt, G.F.R.) was spread as a slurry with resin-filtered water as a 250-μm thick layer on 20 × 20 cm glass plates by means of a Desaga spreader (Desaga, Heidelberg, G.F.R.). Solutions of salts were used to make the adsorbent slurry when the layers (see below) were impregnated with alkaline salts. The plates, activated at 120° for 30 min, were kept in a desiccator over self-indicating silica gel. Before use the silica gel layer was divided into parallel strips approximately 2 cm wide using a round-ended glass rod, and a line was drawn across the top of the plate, about 1 cm from the edge, to mark the solvent front. Samples were applied with pasteur pipettes in 1- to 5-μg quantities; standard oestrogens were

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dissolved in acetone, cholesterol in benzene, and all other steroids in ethanol. All free steroids were obtained from Sigma (St. Louis, Mo., U.S.A.) [6,7-$^3H_2$]Oestrone (6.88 Ci/mmole in benzene) and 17β-[6,7-$^3H_2$]oestradiol (32.7 Ci/mmole in ethanol) were supplied by the Radiochemical Centre (Amersham, Great Britain). Oestrone-3-monochloroacetate (0.52 Ci/mmole) and oestradiol-3,17β-dimonochloroacetate (0.31 Ci/mmole) were prepared from the tritiated oestrogens.

Solvents (AnalaR from BDH, Poole, Great Britain) used for chromatogram development were purified immediately before use as follows: hexane, benzene, diethyl ether, and ethyl acetate according to the methods of Bush[29], chloroform according to the method of Goodspeed and Millson[30], and tetrahydrofuran according to the method of Aakvaag and Eik-Nes[31]. All other solvents were used without further purification.

Chromatograms were developed in glass chambers with filter paper linings in which the developing solvents had been shaken and left at least 2 h to equilibrate. Spots were visualised under UV light, then the plates were sprayed with 50% aqueous sulphuric acid and heated at 70° for 30 min[31,32]; if necessary they were again viewed under UV light.

Steroids were eluted from the silica gel by scraping the spot-containing adsorbent —located by radioactivity scanning— on to a small square of paper about 5 × 5 cm which had a fold down the middle —pen recorder paper was ideal for this as it is very smooth. The adsorbent was transferred to a Pyrex column (1 cm I.D.) with a G-3 sintered glass disc at the base (Jobling, Sunderland, Great Britain). After tapping the adsorbent into a packed form, the chosen solvent was passed through in five 2-ml volumes and all the effluent collected.

Scintillation counting was performed with a Packard Model 3003 Tri-Carb scintillation counter. The scintillator was butyl phenyl biphenyloxadiazole (Ciba, Horsham, Great Britain) in toluene–ethanol (2:1) and efficiencies were calculated using tritiated toluene as an internal standard.

Separation of oestrone, 17β-oestradiol and cholesterol

Solvent systems in different proportions were tested for their ability to separate these compounds (Table I). In another experiment the silica gel slurry was made with either 0.1 *M* sodium bicarbonate or 0.2 *M* sodium carbonate solution instead of resin-filtered water to see if alkaline salts retarded the oestrogens, a set of non-impregnated plates being developed as controls (Table I).

Separation of oestrogens from their derivatives

The separation of acetyl, monochloroacetyl, methyl ether, bromomethyldimethylsilyl ether, *p*-bromobenzoyl and pentafluorophenylhydrazone derivatives from oestrogens with benzene–ethyl acetate (6:1) and (5:1) systems was investigated (Table II).

Effect of temperature on oestrogen and oestrogen monochloroacetate mobilities

It was noted that mobilities and separations varied according to whether it was a cold or a hot day, thus a number of developments were carried out with the chambers at different temperatures (5°–30°). After chamber equilibration overnight, chromatograms were developed with benzene–ethyl acetate (2:1). Oestriol was included in the study to find the effect of temperature on a compound with a low mobility (Table III).

Elution of oestrogens and oestrogen monochloroacetates from silica gel

Four samples of each carrier-free tritiated oestrogen (about 3 × 10^5 dpm) were applied as separate spots to a TLC plate. After drying, each spot was scraped into a column and eluted with a solvent mixture. The following solvent mixtures were used: (a) diethyl ether, (b) toluene–methanol (19:1), (c) chloroform–methanol (2:1), and (d) chloroform–methanol (3:1). This was repeated with samples of tritiated oestrogen monochloroacetates (about 1 × 10^{-5} dpm) using the following solvent mixtures: (a) chloroform–methanol (9:1), (b) chloroform–diethyl ether (1:3), and (c) benzene–ethyl acetate (19:1).

RESULTS AND DISCUSSION

Table I shows that benzene–ethyl acetate (2:1) and (3:1) systems give separations of oestrone and 17β-oestradiol of 0.2 R_F units. Mobilities were measured in triplicate and standard deviations were less than, or equal to, ± 0.02. While relative mobilities were the same, R_F values were at variance with those found by others[3,4,7,13,33] possibly because of effects of temperature[34,35], humidity[36], amounts of sample[37,38], source of the silica gel[37], and definition of R_F[37] —here measured as the relative distance, travelled by the solute and the solvent front. The loss of sharpness and tailing observed by Takeuchi[7] after development with hexane–ethyl acetate systems was not found.

TABLE I

MOBILITIES OF OESTRONE, 17β-OESTRADIOL AND CHOLESTEROL ON SILICA GEL

Developing solvent system	*Oestrone*	*17β-Oestradiol*	*Cholesterol*
Benzene–ethyl acetate (2:1)	0.55	0.35	—
Benzene–ethyl acetate (3:1)	0.45	0.26	—
Benzene–ethyl acetate (5:1)	0.37	0.19	—
Chloroform–diethyl ether (5:2)	0.64	0.36	0.44
Hexane–chloroform (4:1)	0.00	0.00	0.02
Hexane–dichloromethane (1:2)	0.16	0.05	0.22
Hexane–methanol (4:1)	0.02	0.06	0.34
Hexane–ethyl acetate (5:2)	0.28	0.15	0.40
Hexane–ethyl acetate (5:2)*	0.17	0.09	0.28
Hexane–ethyl acetate (5:2) + 0.007 volumes 15 *N* NH_4OH	0.13	0.06	0.30
Hexane–ethyl acetate (1:1)	0.52	0.38	0.58
Hexane–ethyl acetate (1:1)*	0.54	0.43	0.54
Hexane–ethyl acetate (1:1)**	0.41	0.30	0.46

* Silica gel plates prepared with 0.1 *M* sodium bicarbonate solution.

** Silica gel plates prepared with 0.2 *M* sodium carbonate solution.

Cholesterol and oestrone were not separated throughout a range of hexane–ethyl acetate systems. However, Neher[39] has shown that mobilities and relative solubilities of steroids may vary widely throughout a certain equieluotropic range of solvent systems, thus the search for a suitable solvent had been on a trial-and-error basis. Only hexane–methanol (4:1) (Table I) gave an adequate separation of cholesterol from the oestrogens but did not separate oestrone from 17β-oestradiol.

A solvent system containing a base (ammonium hydroxide) was tried to see if it changed the relative mobilities (Table I). All the steroids were retarded.

The presence of alkaline salts (Table I) was also expected to modify the mobilities of the acidic oestrogens. Sodium bicarbonate caused slightly increased mobilities of all the steroids with hexane–ethyl acetate (1:1) but caused decreased mobilities with hexane–ethyl acetate (5:2) as did sodium carbonate with hexane–ethyl acetate (1:1) (Table I).

Separations of 0.18 and 0.52 were obtained between oestrone and 17β-oestradiol and their monochloroacetates with benzene–ethyl acetate (6:1). This system gave a better separation of the other derivatives from their parent oestrogens than benzene–ethyl acetate (5:1), except in the case of oestrone-3-methyl ether (Table II). Mobilities obtained here were slightly lower than those reported by Van der Molen and Groen[13] and Rado *et al.*[14].

TABLE II

MOBILITIES OF OESTROGENS AND THEIR DERIVATIVES ON CHROMATOGRAMS DEVELOPED WITH BENZENE–ETHYL ACETATE

Figures in brackets denote the differences in mobility between the derivative and the parent oestrogen.

Steroid derivative	*R_F value*	
	Benzene–ethyl acetate (6:1)	*Benzene–ethyl acetate (5:1)*
Oestrone	0.30	0.34
Oestrone-3-methyl ether	0.41 (0.11)	0.50 (0.16)
Oestrone-3-acetate	0.49 (0.19)	0.51 (0.17)
Oestrone-3-monochloroacetate	0.48 (0.18)	0.50 (0.16)
Oestrone-3-*p*-bromobenzoate	0.53 (0.23)	0.53 (0.19)
Oestrone-3-bromomethyldimethylsilyl ether	—	0.51 (0.17)
Oestrone-17-pentafluorophenylhydrazone	0.49 (0.19)	0.52 (0.18)
17β-Oestradiol	0.16	0.16
Oestradiol-3,17β-diacetate	0.56 (0.40)	0.56 (0.40)
Oestradiol-3,17β-dimonochloroacetate	0.66 (0.50)	0.67 (0.51)
Oestradiol-3,17β-di-*p*-bromobenzoate	0.61 (0.45)	0.60 (0.44)
Oestradiol-3,17β-dibromomethyldimethylsilyl ether	0.62 (0.46)	0.52 (0.36)

As the temperature was reduced, plates took longer to run and R_F values increased. The lower the temperature, the better was the separation of the oestrogens from each other (Table III). The separation of oestrogens from their chloroacetates also varied with temperature: 17β-oestradiol was best separated at 10° while oestrone separation reached a maximum at 25°.

Almost complete recoveries from silica gel of oestrone, 17β-oestradiol and their chloroacetates were obtained. The chloroform–methanol systems were most effective but diethyl ether gave a lower recovery of oestrogens and benzene–ethyl acetate (19:1) a lower recovery of their monochloroacetates. Recoveries of oestrogens were rather higher than those reported by Attal *et al.*[25], Masaracchia and Gawienowski[26] and Vandenheuvel[28], but differences were within the error of the counting. Chloroform–

TABLE III

EFFECT OF TEMPERATURE ON CHROMATOGRAPHIC SEPARATIONS ON SILICA GEL

Solvent system: Benzene–ethyl acetate (2:1). (a) Oestradiol-3,17β-dimonochloroacetate, (b) oestrone-3-monochloroacetate, (c) oestrone, (d) 17β-oestradiol, and (e) oestriol.

Temperature (°C)	*Running time (min)*	*R_F value*				
		a	*b*	*c*	*d*	*e*
5	39.2	0.76	0.67	0.56	0.36	0.17
10	36.4	0.75	0.65	0.52	0.33	0.16
15	33.6	0.71	0.63	0.48	0.31	0.14
20	30.2	0.68	0.59	0.43	0.28	0.13
25	26.0	0.63	0.54	0.37	0.24	0.11
30	19.2	0.52	0.44	0.28	0.18	0.08

methanol (3:1) rather than (2:1) was used for routine elution of plasma oestrogens in order to reduce possible breakdown of steroids on the silica gel and also the elution of small silica gel particles. Toluene–methanol (19:1) was difficult to evaporate and therefore not used in practice. Oestrogen monochloroacetates were eluted routinely with chloroform–methanol (9:1). Chloroform–diethyl ether (1:3) was not used as we found difficulty in purifying the diethyl ether adequately for the gas–liquid chromatographic measurements.

The solvent systems found optimal for the elution were used for sample application as they readily dissolved the oestrogens or their derivatives.

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