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GEL CHROMATOGRAPHY OF STEROID OESTROGENS ON SEPHADEX LH-20

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SUMMARY

The behaviour of 23 steroid oestrogens on Sephadex LH-20 using six solvent systems was investigated. The fractions eluted by gel column chromatography were analysed by thin-layer chromatography and spectrometry.

The method was used for the analysis of the radiochemical purity of labelled compounds by isotopic dilution.

INTRODUCTION

Gel chromatography on lipophilic Sephadex has been used to separate several classes of steroids (for a review, see Thunberg[1]). In spite of its use for the purification and separation of the most common oestrogens, *viz.*, oestrone, 17β-oestradiol and oestriol[2–4], there has been no systematic investigation of the elution behaviour of a large number of oestrogens.

During investigations into the metabolism of steroid oestrogens in mammalian tissue, we employed column chromatography on Sephadex LH-20 for the purification of samples and also for the identification of isolated metabolites[5–8]. The gel column chromatographic behaviour of 23 steroid oestrogens in the system developed for these investigations and the application of this method for the determination of radiochemical purity is described in this paper.

MATERIALS AND METHODS

Sephadex LH-20, a hydroxypropylated derivative of Sephadex G-25 (ref. 9) (25–100 mesh), was obtained from Pharmacia, Uppsala, Sweden. The steroids used were purchased from commercial sources or were gifts from colleagues (see Table I). The abbreviations used in Table I and Fig. 1 are as follows: E = 1,3,5(10)-oestratrien-3-ol; E_1 = oestrone; E_2 = oestradiol (17β if not otherwise stated); and E_3 = oestriol. [4-^{14}C]Oestrone was obtained from NEN Chemicals (NEC-512).

The following solvent systems were used: S-1, *n*-hexane–chloroform–methanol (80:10:10); S-2, isooctane–chloroform–methanol (80:10:10); S-3, cyclohexane–

chloroform–methanol (80:10:10); S-4, cyclohexane–ethyl acetate–methanol (80:10:10); and S-6, *n*-heptane–chloroform–methanol (88:10:2).

Glass-columns, 30 cm × 1 cm I.D., with PTFE stopcocks were filled with 4–8 g of Sephadex LH-20 that had been allowed to swell for several hours in the solvent system employed. The Sephadex slurry that remained on the glass walls was rinsed down with the same solvent. The upper surface of the gel bed was protected with a layer of fine sand. The steroid sample was dissolved in 0.5 ml of the solvent employed and applied with a capillary to the surface of the packing. Elution was started by initially adding the solvent system in small amounts. Fractions of 2.5 or 5.0 ml were collected automatically in a fraction collector (LKB) at a flow-rate of 30–60 ml/h.

The eluted fractions were analyzed by thin-layer chromatography (TLC) on silica gel G (ref. 10) and the spots of the steroids rendered visible by means of the Folin–Ciocalteu reaction or the anisaldehyde–sulphuric acid–acetic acid reaction[11]. In the example given in Fig. 2, the system cyclohexane–ethyl acetate–ethanol (45:45:10) was used.

The identity of a radioactive metabolite was determined by gel column chromatography of the labelled material after the addition of an amount of the corresponding authentic standard (100–500 μg). According to the polarity of the steroid in the solvent system used, 1–2.5-ml (for more lipophilic steroids) or 2.5–10-ml (for more polar compounds) fractions were collected. The specific activity in each fraction was calculated by measuring the radioactivity in a liquid scintillation counting apparatus until the standard deviation was less than 2.5, and by determination of the weight of the steroid or its corresponding chromogenicity in sulphuric acid (Kober reaction). The concentration of sulphuric acid and the wavelength used for spectrophotometric assessments depended on the type of oestrogen. The conditions for the Kober reaction have been described elsewhere[12]. The volume of the aliquot used for the determination of the weight of the steroid depended on the chromogenicity of the steroid oestrogens in the Kober reaction.

Oestrogens that show low sulphuric acid chromogenicity, such as 6-oxygenated compounds, can be measured by gas chromatography. The proof of purity was accepted when a constant specific activity (dpm/μg, cpm/μg or absorbance) expressed as the logarithm of the specific activity (log S.A.) was found in all of the fractions. Minute amounts of Sephadex LH-20 eluted with the solvent systems did not interfere in the procedure.

In the example given in Table II, [4-^{14}C]oestrone (25,000 cpm) with 200 μg of recrystallized oestrone added was chromatographed on 4 g of Sephadex LH-20 using solvent system S-6. A 0.2-ml volume of each 10-ml fraction was used for the radioactivity determination, whereas the weight of the steroid was expressed by the mean value of the absorbances of two samples found after the development of the sulphuric acid chromogenicity (Kober reaction) according to Brown[13].

RESULTS AND DISCUSSION

Table I summarizes the behaviour (effluent volume in millilitres) of 23 free steroid oestrogens on gel column chromatography using Sephadex LH-20 and six solvent systems. As reported for other groups of steroids[14,15], monohydroxy-mono-

TABLE I

CHROMATOGRAPHIC BEHAVIOUR OF FREE STEROID OESTROGENS ON SEPHADEX LH-20 (25–100 MESH) USING SIX SOLVENT SYSTEMS

Fractions of 2.5–5.0 ml (S-1–S-5) or 10 ml (S-6) obtained with a flow-rate of 20–25 ml/h were analysed by TLC on silica gel G. Results are effluent volumes in millilitres.

*Steroid**	*Solvent system*					
	S-1 (4 g)	*S-2 (4 g)*	*S-3 (4 g)*	*S-4 (4 g)*	*S-5 (8 g)*	*S-6 (4 g)*
$\Delta^{1,6}$E (a)		15– 25	10– 30	5– 15		10– 60
E_1 (b)	30– 45	30– 50	20– 35	15– 30	30– 50	80–160
$\Delta^{7}E_1$ (c)		40– 60	25– 40	15– 30		110–200
$\Delta^{6,8}E_1$ (c)		45– 70	30– 50	20– 35		180–300
$\Delta^{6}E_1$ (c)		40– 60	25– 45	20– 35		120–210
E_2-17α (b)	65– 90	70–100	40– 65	20– 45		
E_2-17β (b)	65– 95	70–100	45– 65	25– 30	80–110	
$\Delta^{6}E_2$ (c)	80–115	95–130	50– 85	25– 60		
6-oxo-E_2 (c)	85–125	100–135	55– 90	35– 75	85–115	
16-oxo-E_1 (a)		95–125	55– 80	35– 60		
16α-ol-E_1 (d, c)	95–145	115–155	60– 95	35– 80	80–100	
16-oxo-E_2 (d)	105–140	120–155	70– 95	45– 85	80–105	
17-epi-E_3 (c)	110–170	140–180	75–110	40– 80	155–190	
16-epi-E_3 (c)	125–170	140–185	80–110	45– 85	160–210	
E_3 (b)	170–220	180–250	110–155	80–130	260–355	
6α-ol-E_2 (c)	195–245	205–275	115–165	85–145	220–330	
7α-ol-E_2 (e)		230–300	145–205	100–155		
6-oxo-E_3 (c)	210–260	235–290	145–185	115–165	290–380	
16,17-epi-E_3 (f)	225–290	245–310	140–200	85–145	320–425	
15α-ol-E_2 (g)	270–335	270–370	160–230	120–175	310–395	
15α-ol-E_3 (c)	310–395	340–440	195–255	140–215		
6α-ol-E_3 (f)	415–570	445–545	255–395	270–415		
2-ol-E_3 (c)		500–650	310–400	235–325		

* Source: (a) Ikapharm, Ramat-Gan, Israel; (b) Schering, Berlin, G.F.R.; (c) Steraloids, Pawling, N.Y., U.S.A.; (d) Dipl. Eng. I. Könyves, Hälsingborg, Sweden; (e) Prof. Dr. H. Breuer, Bonn, G.F.R.; (f) Sigma, St. Louis, Mo., U.S.A.; (g) Dr. P. Diassi, Squibb & Sons, New Brunswick, N.J., U.S.A.

ketonic (oestrone), dihydroxy- (17β-oestradiol), dihydroxy-monoketonic (6-oxo- or 16-oxo-oestradiol), and trihydroxy- (2-, 6α- or 15α-hydroxyoestradiol) steroids were completely separated from each other.

The introduction of unsaturated bonds in the B ring retards the elution of the steroid from the gel, which permits a partial separation to be achieved between oestrone, equilin (Δ^{7}-oestrone) and equilenin ($\Delta^{6,7}$-oestrone) in the system S-6 or between 17β-oestradiol in solvent systems S-2, S-3 and S-4. Steroids with double-bond isomerism (Δ^{6}-oestrone and Δ^{7}-oestrone) and 17-epimeric oestradiols remained unresolved in all of the systems tested.

In the group of oestriols, partial separations were obtained for the pairs 6α–7α-hydroxyoestradiol and oestriol–16,17-epioestriol. Oestriol and 15α-hydroxy-oestradiol were completely separated from each other in systems S-1, S-2 and S-3. In all of the systems, epimeric oestriols with a transoid structure (oestriol and 16,17-epioestriol) were separated from their cisoid epimers (16-epi- and 17-epioestriol); however, this last group resisted all attempts at separation.

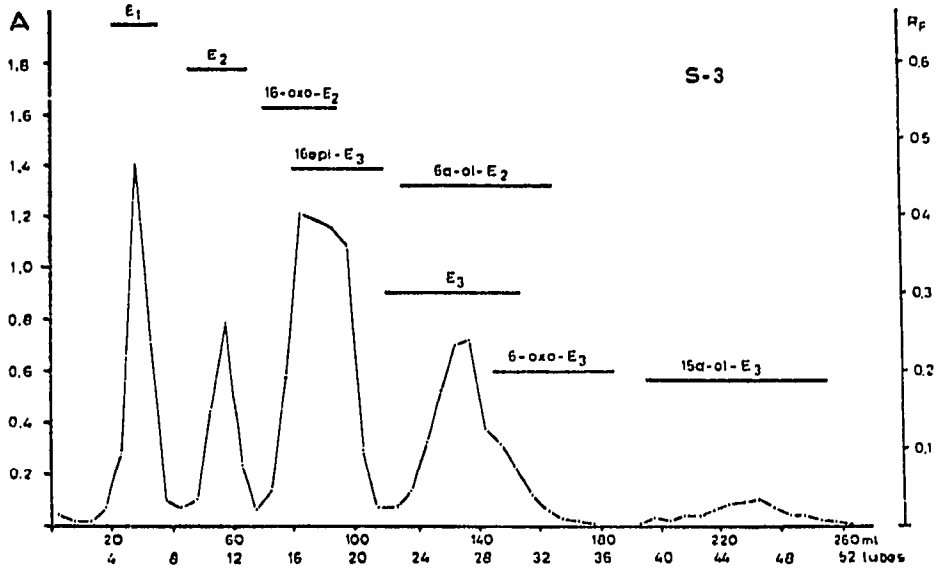

Fig. 1. Sephadex gel chromatography (4 g) of a mixture of eight steroid oestrogens in system S-3. Aliquots of the 2.5-ml fractions eluted from the column were analyzed by TLC and by spectrometry following the Kober reaction.

Fig. 1 shows the chromatographic behaviour of a mixture of eight steroid oestrogens on Sephadex gel developed with system S-3. The fractions eluted from the column were analyzed at the same time by TLC on silica gel G, and by spectrometry using the sulphuric acid colour reaction. This example shows the advantages of the investigation of the eluates by two different procedures. Steroid groups that are not resolved by gel chromatography in the system used as eluent, for instance 16-oxo-oestradiol–16-epioestriol and 6α-hydroxyoestradiol–oestriol, could be detected only by TLC. It is interesting to note the lack of chromogenicity of 6-oxo-oestriol with sulphuric acid.

Sephadex gel column chromatography can be employed for the analysis of the radiochemical purity of a radioactively labelled compound by calculating its specific activity in the different fractions eluted from the gel. Table II and Fig. 2 give an example of the application of this procedure to the identification of labelled compounds. Table II gives results for the determination of the radiochemical purity of [4-^{14}C]oestrone by Sephadex LH-20 using system S-6 as eluent; 95% of the radioactivity (23,727 cpm) was found in fractions 8–17, corresponding to an eluent volume between 70 and 170 ml. A relative specific activity was calculated for the fractions in which the absorbance values were higher than 0.01, and in which the radioactivity was measured with a standard deviation of less than or equal to 2.5%. The mean of the log S.A. calculated for the fractions that fulfilled these requirements (9–16) showed a standard deviation of about 10%, indicating that these values show the radiochemical purity of the steroid investigated.

Another aliquot of [4-^{14}C]oestrone (30,000 cps) to which 150 μg of unlabelled steroid were added was chromatographed on 8 g of Sephadex LH-20 in system S-5 (column size 19.8 × 1.2 cm), and 98% of the radioactivity was found in tubes 22–30,

TABLE II

DETERMINATION OF THE RADIOCHEMICAL PURITY OF [4-^{14}C]OESTRONE BY GEL COLUMN CHROMATOGRAPHY ON SEPHADEX LH-20

Oestrone (200 μg) and [4-^{14}C]oestrone (25,000 cpm) were chromatographed on 4 g of Sephadex LH-20 in system S-6; 2-ml aliquots of each 10-ml fraction were used for radioactive counting and oestrogen determination (Kober reaction).

Effluent fraction no. (10-ml fractions)	*Absorbance (A)*			*Radioactivity*		*Relative specific activity = cpm/1000·A**	*Log S.A.*
	a	*b*	*Mean*	*cpm*	*S.D.*		
8	0.005	0.005	0.005	41.0	2.5		
9	0.035	0.032	0.033	100.7	2.5	3.05	0.4843
10	0.075	0.075	0.075	225.1	1.5	3.00	0.4771
11	0.115	0.115	0.115	332.1	1.5	2.89	0.4609
12	0.140	0.152	0.146	413.2	1.5	2.83	0.4518
13	0.327	0.325	0.326	935.6	1.0	2.87	0.4579
14	0.580	0.570	0.575	1576.3	0.7	2.74	0.4378
15	0.340	0.340	0.340	913.3	1.0	2.69	0.4298
16	0.080	0.085	0.082	180.9	1.5	2.21	0.3444
17	0.010	0.015	0.012	27.3	3.5		
						Mean: 2.79 ± 0.26	0.4430 ± 0.0438

* Calculated for absorbance (A) values higher than 0.005 and cpm values with an S.D. of not more than 2.5%.

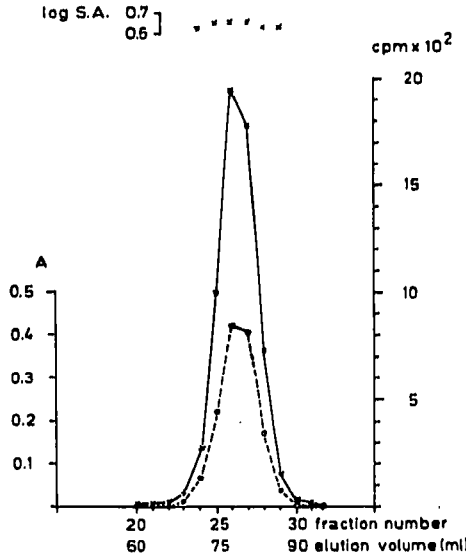

Fig. 2. Determination of the radiochemical purity of [4-^{14}C]oestrone by gel column chromatography on Sephadex LH-20 in system S-5.

corresponding to an eluent volume of 66–90 ml. Then 0.6-ml aliquots of each 3-ml fraction were analyzed for radioactivity (cpm) and for weight of oestrogen (expressed as absorbance units). As can be seen in Fig. 2, the relationship between the logarithm of the relative specific activity, calculated as the ratio of radioactivity (cpm) to absorbance, and the fraction number is linear and can be used in the assay of the radiochemical purity of the chromatographed compound.

This method is similar to those used in counter-current distribution[16], column adsorption chromatography[17] and TLC[18,19] and can be used even when only 1000 dpm of isotopic material is available[20]. However, several epimeric or closely related compounds cannot be separated by Sephadex gel chromatography; therefore, this procedure must be applied with caution to studies of steroid metabolism.

Even if more than one chromatographic system is used, it is necessary to point out that the radiochemical purity of an isolated material can never be accepted beyond any doubt. Therefore, the identification of such a compound must be carried out in combination with other available methods such as radio-gas chromatography.

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