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Note

Chromatography of C_{18}, C_{19} and C_{21} steroids on Sephadex LH-20

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The usefulness of chromatography on lipophilic Sephadex for the purification and separation of steroids is well established[1–3] and has been applied to a variety of separation problems including conjugated plasma steroids[1,2,4–10], conjugated urinary steroids[11], unconjugated plasma steroids[12–18] and unconjugated urinary steroids[14,19–22].

In our laboratory, Sephadex LH-20 (5 g) has been used over the past few years especially for the purification of samples before identification by gas chromatography–mass spectrometry, particularly urinary steroids[22], with chloroform–heptane–methanol (5:5:1) as the eluent, derived from that advocated by Nyström and Sjövall[3].

In this paper, we present results obtained when our method was applied to the separation of steroid hormones of general clinical interest (*e.g.*, cortisol and aldosterone) and of some steroids of more specific interest, especially in relation to our research on the metabolism of 21-deoxysteroids during human gestation (*e.g.*, progesterone and 16 α-hydroxyprogesterone).

We describe a gel chromatographic study carried out on 35 reference steroids. First we used smaller amounts of Sephadex LH-20 (usually 1 g) than in previous studies, and second we modified the polarity of the system in order to reduce the elution volumes to 30 ml. These two modifications led to an improvement in the resolution of the chromatographic profiles and made possible some usually very difficult or impossible separations with Sephadex LH-20.

The following trivial names and abbreviations are used: 6α-OHP = 6α-hydroxy-4-pregnene-3,20-dione; 6β-OHP = 6β-hydroxy-4-pregnene-3,20-dione; 6β-OHf = 6β,11β,17,21-tetrahydroxy-4-pregnene-3,20-dione; aldosterone = 11β,21-dihydroxy-4-pregnene-3,18,20-trione; T = 17β-hydroxy-4-androsten-3-one; androsterone = 3α-hydroxy-5α-androstan-17-one; epiandrosterone = 3β-hydroxy-5α-androstan-17-one; etiocholanolone = 3α-hydroxy-5β-androstan-17-one; 11-oxoandrosterone = 3α-hydroxy-5α-androstane-11,17-dione; 11-oxoepiandrosterone = 3β-hydroxy-5α-androstane-11,17-dione; 11-oxoetiocholanolone = 3α-hydroxy-5β-

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androstane-11,17-dione; 11β-hydroxyandrosterone = 3α,11β-dihydroxy-5α-androstan-17-one; 11β-hydroxyetiocholanolone = 3α,11β-dihydroxy-5β-androstan-17-one; 11β-hydroxyepiandrosterone = 3β,11β-dihydroxy-5α-androstan-17-one; DOC = 21-hydroxy-4-pregnene-3,20-dione; S compound = 17,21-dihydroxy-4-pregnene-3,20-dione; B = 11β,21-dihydroxy-4-pregnene-3,20-dione; E = 17,21-trihydroxy-4-pregnene-3,11,20-trione; F = 11β,17,21-trihydroxy-4-pregnene-3,20-dione; P = 4-pregnene-3,20-dione; 20α-DHP = 30α-hydroxy-4-pregnen-3-one; 20β-DHP = 20β-hydroxy-4-pregnen-3-one; 6-oxoP = 4-pregnene-3,6,20-trione; 11β-OHP = 11β-hydroxy-4-pregnene-3,20-dione; 11-oxoP = 4-pregnene-3,11,20-trione; 16-oxoP = 4-pregnene-3,16-20-trione; 16α-OHP = 16α-hydroxy-4-pregnene-3,20-dione; 17-OHP = 17-hydroxy-4-pregnene-3,20-dione; DHA = 3β-hydroxy-5-androsten-17-one; THS = 3α,17,21-trihydroxy-5β-pregnan-20-one; E4 = 1,3,5(10)-oestratriene-3,15α,16α,17β-tetrol.

EXPERIMENTAL

Steroids

Cholesterol, 15α-hydroxyestriol (E4), 6α-hydroxyprogesterone (6α-OHP), 6β-hydroxyprogesterone (6β-OHP), 4-pregnene-3,6,20-trione (6-oxoP), 4-pregnene-3,16,20-trione (16-oxoP), 6β-hydroxycortisol (6β-OHF) and aldosterone were obtained from Steraloids (Wilton, NH, U.S.A.). All other reference C_{18}, C_{19} and C_{21} steroids were purchased from Makor Chemicals (Jerusalem, Israel). Chromatographic standards were stored as 1 mg/ml stock solutions in methanol.

Chemicals

Analytical-reagent grade solvents were purchased from Merck (Darmstadt, F.R.G.) and Mallinckrodt (St. Louis, MO, U.S.A.). Sephadex LH-20 was obtained from Pharmacia (Uppsala, Sweden). The solvent mixtures used for Sephadex LH-20 chromatography were S1 = chloroform–heptane–methanol (5:5:1), S2 = chloroform–heptane–methanol (5:5:2) and S3 = chloroform–heptane–methanol (10:10:1).

Preparation of Sephadex LH-20 columns

Glass columns (Spiral, Dijon, France), 30 cm × 0.4 cm I.D., with a 25 ml solvent reservoir and a Teflon stopcock, were used.

Sephadex LH-20 (1 g per column) was suspended overnight in 10 ml of solvent mixture, then transferred into the column. After complete sedimentation of Sephadex, the column was ready for use.

Chromatography

All analyses were performed in triplicate at room temperature with S1.

A volume of 0.5–2.5 ml of methanolic steroid working solution 10 μg/ml) was evaporated to dryness and the residue, dissolved in 2 × 0.1 ml of S1, was transferred to the Sephadex LH-20 column.

Steroids were eluted with S1. During chromatography a pressure of nitrogen was applied to the column to maintain a flow-rate of approximately 5 ml/h. The collected fractions (1 ml) were evaporated to dryness under nitrogen.

TABLE I

ELUTION VOLUMES AND RECOVERIES OF CHOLESTEROL AND REFERENCE C_{18} STEROIDS CHROMATOGRAPHED ON SEPHADEX LH-20 USING S_1 (n = 3)

Reference steroid	*Elution volume (ml)*	*Recovery (%)*
Cholesterol	2–3	95 ± 3
Oestrone	4–5	96 ± 2
Oestradiol	8–10	87 ± 6
Oestriol	16–19	84 ± 6
15α-Hydroxyoestriol	24–30	76 ± 8

The elution volumes and the recoveries of the Sephadex LH-20 chromatography were determined using the colorimetric method of Zimmermann[23] for DHA and saturated 17-oxosteroids, the method of Porter and Silber[24] for THS and the method of Kober[25] for the estrogens. The 3-oxo-4-ene-steroids were detected by UV spectrophotometry (240 nm). A calibration graph, obtained for each reference compound, was used for recovery measurements.

Depending on results for each compound (S1, 1-ml fraction), three to five selected steroids were analysed simultaneously. In addition to solving certain specific separation problems, the volume of fractions collected with S1 was reduced (0.25 and 0.5 ml) and S2 and S3 were used.

RESULTS AND DISCUSSION

Cholesterol and C_{18} steroids

Elution volumes and recoveries for cholesterol, oestrone (E1), oestradiol-17β (E2), oestriol (E3) and 15α-hydroxyoestriol are given in Table I. Using S1, a complete separation of the four estrogens was obtained. The recoveries were consistent with those obtained by other workers[17,26]. Using S2, the elution volumes of E1, E2 and

TABLE II

ELUTION VOLUMES AND RECOVERIES OF C_{19} STEROIDS CHROMATOGRAPHED ON SEPHADEX LH-20 USING S_1 (n = 3)

Underlined values are those of the major elution fraction.

Reference steroid	*Elution volume (ml)*	*Recovery (%)*
4-Androstene-3,17-dione	3	91 ± 3
Testosterone	3–4	96 ± 2
Androsterone	3–4	90 ± 3
Epiandrosterone	3–4	76 ± 5
Dehydroepiandrosterone	3–4	78 ± 4
Etiocholanolone	4–5	96 ± 2
11-Oxoepiandrosterone	4–5	91 ± 4
11-Oxoandrosterone	4–5	89 ± 5
11-Oxoetiocholanolone	4–5	82 ± 3
11β-Hydroxyandrosterone	5–6	97 ± 2
11β-Hydroxyetiocholanolone	5–6	86 ± 5
11β-Hydroxyepiandrosterone	5–6	95 ± 4

E3 were 3–4.5 ml, 4.5–6.5 ml and 6.5–9.5 ml, respectively. With 0.5-ml collected fractions, complete separation of each of the three components was achieved. Under these conditions, the chromatographic yields were virtually the same as those obtained using S1 (Table I).

C_{19} steroids

The chromatographic parameters (S1, 1-ml fraction) of 4-androstene-3,17-dione, testosterone (T) and nine saturated 17-oxosteroids were determined.

4-Androstene-3,17-dione, testosterone (T) and androsterone were eluted principally in the Sephadex LH-20 fraction 3, epiandrosterone and DHA in fractions 3 and 4, etiocholanolone in fraction 4, 11-oxoepiandrosterone in fractions 4 and 5, 11-oxoandrosterone, 11-oxoetiocholanolone and 11β-hydroxyandrosterone in fraction 5, 11β-hydroxyetiocholanolone in fractions 5 and 6 and 11β-hydroxyepiandrosterone in fraction 6. Table II gives the elution volumes and recoveries of these C_{19} steroids.

Under these conditions (S1, 1-ml fraction), 4-androstene-3,17-dione and T were not separated. However, using S3 and with 0.25-ml fractions complete separation was achieved (Fig. 1).

Except for DHA, the saturated C_{19} steroids analysed are representative of the urinary metabolites of testosterone, 4-androstene-3,17-dione and 11β-hydroxy-4-androstene-3,17-dione. Under the standard conditions, and with 0.25-ml fractions, the results obtained show that the main 11-deoxy-17-oxosteroids (androsterone, etiocholanolone, epiandrosterone and DHA) eluted principally in fractions 3 and 4, the 11,17-dioxo-C_{19} steroids (11-oxoandrosterone, 11-oxoetiocholanolone) eluted principally in fraction 5 but the 11β-hydroxy-17-oxosteroids (11β-hydroxyandrosterone, 11β-hydroxyetiocholanolone) were difficult to separate. However, this difficulty may

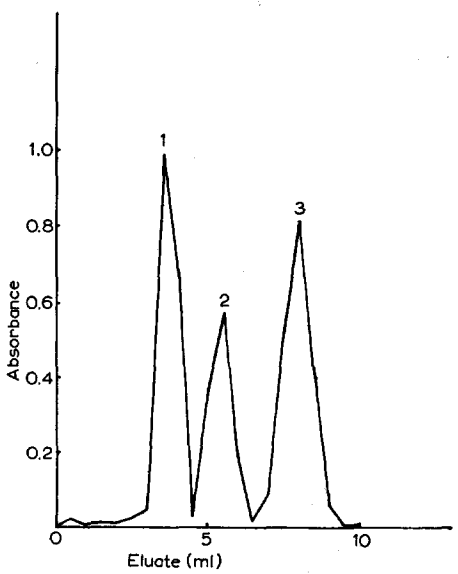

Fig. 1. Chromatogram of estrogens obtained using solvent system S_2. The volume of each collected fraction was 0.5 ml. Peaks: 1 = oestrone; 2 = oestradiol-17β; 3 = oestriol.

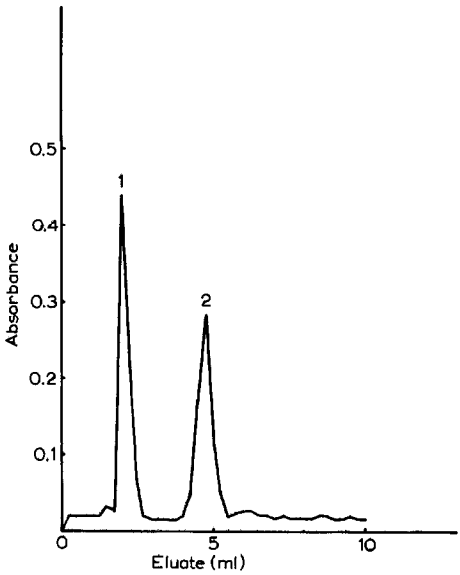

Fig. 2. Chromatogram of 4-androstene-3,17-dione (peak 1) and testosterone (peak 2) obtained using solvent system S_3. The volume of collected fractions was 0.25 ml.

be overcome by using gas-phase analysis (gas–liquid chromatography and gas chromatography–mass spectrometry) of the 17-oxosteroids [analysed as their methoxylamine-trimethylsilyl (MO-TMS) derivatives] eluted in fractions 3–6. Under these conditions, a specific study of urinary 17-oxosteroids may be undertaken when required.

21-Deoxysteroids

In addition to P, 20α-dihydroprogesterone (20α-DHP) and 20β-dihydroprogesterone (20β-DHP), six oxygenated derivatives of P, namely 6-oxoprogesterone

TABLE III

ELUTION VOLUMES AND RECOVERIES OF REFERENCE 21-DEOXYSTEROIDS CHROMATOGRAPHED ON SEPHADEX LH-20 USING S_1 (n = 3)

Underlined values are those of the major elution fraction.

Steroid	*Elution volume (ml)*	*Recovery (%)*
Progesterone	2–3	94 ± 3
6-OxoP	2–3	91 ± 3
11-OxoP	2 3 4	96 ± 2
16α-OHP	2–3–4	79 ± 4
16-OxoP	2–3–4	90 ± 4
20α-DHP	3–4	91 ± 3
20β-DHP	3–4	85 + 4
6α-OHP	3–4	–
6β-OHP	3–4	–
11β-OHP	3–4	–
17-OHP	3–4	92 ± 4

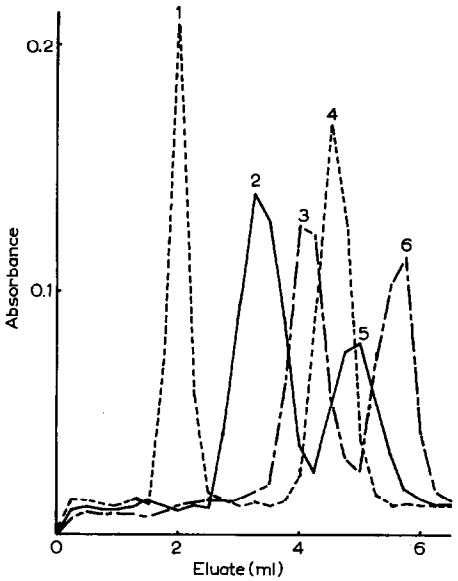

Fig. 3. Elution pattern of P (peak 1), 20α- and 20β-DHP (peak 2), 17α-OHP (peak 3), 6α- and 6β-OHP (peak 4), 11βOH-P (peak 5) and 16α-OHP (peak 6) obtained using solvent system S_3.

(6-oxoP) 6α- and 6β-hydroxyprogesterone, 11-oxoprogesterone (11-oxoP), 11β-hydroxyprogesterone (11β-OHP), 16-oxoprogesterone (16-oxoP), 16α-hydroxyprogesterone (16α-OHP) and 17-hydroxyprogesterone (17-OHP), were chromatographed on Sephadex LH-20. The results obtained are summarized in Table III.

Under the standard conditions (S1, 1-ml fraction) the separation of these steroids is difficult, as their elution volumes lie between 2 and 5 ml. A better separation is obtained if the collected fraction volume is reduced to 0.25 ml. However, the best resolution for this steroid series was achieved with S3; with 0.25-ml fractions, P, 20-DHP, 17-OHP, and 16α-OHp were separated, but 6α- and 6β-OHP, 11-oxoP and 11β-OHP (eluted between 17-OHP and 16α-OHP) were not separated (Fig. 3).

TABLE IV

ELUTION VOLUMES AND RECOVERIES OF 21-HYDROXYSTEROIDS CHROMATOGRAPHED ON SEPHADEX LH-20 USING S_1 (n = 3)

Underlined values are those of the major elution fraction.

Steroid	*Elution volume (ml)*	*Recovery (%)*
DOC	3–4	90 ± 3
S compound	4–5	91 ± 4
B	4–5–6	85 ± 6
E	4–5–6	93 ± 4
Aldosterone	4–5–6	94 ± 3
THS	6–7–8	77 ± 8
F	8–9–10	92 ± 4
6βOHF	16–18	52 ± 9

21-Hydroxysteroids

The chromatographic characteristics of eight reference compounds, namely deoxycorticosterone (DOC), 11-deoxycortisol (S compound), corticosterone (B), cortisone (E) cortisol (F), 6β-hydroxycortisol, aldosterone and THS were determined. Table IV gives the elution volumes and the recoveries of these reference compounds. Using S1, with the exception of aldosterone, corticosterone and cortisol (eluted in fractions 5 and 6), all other 21-hydroxysteroids were separated.

With S1 (1-ml fraction), S, E, B and aldosterone, eluted in fractions 4–6, were not separated. However, DOC (eluted in fraction 3) and F (eluted principally in fraction 9) were separated from each other and from S compound, E, B and aldosterone. Given the clinical context, this method of separation can be used only for assay of S compound or of DOC, aldosterone and F.

Under the standard conditions (S1, 1-ml fraction), THS is not completely separated from F. THS assay is used principally in the metyrapone test. In this instance, as the urinary excretion of F becomes negligible, the separation problem does not arise. In addition, if necessary, complete separation of these two steroids is achieved using S1 and collecting 0.50-ml fractions.

Finally, 6β-OHF, the elution volume of which lies between 16 and 18 ml, was well separated from all the other 21-hydroxysteroids (Table IV).

CONCLUSION

The elution volumes and chromatographic yields on Sephadex LH-20 of cholesterol and 35 other reference steroids (four oestrogens, twelve C_{19} steroids, eleven 21-deoxysteroids and eight 21-hydroxysteroids) were determined. Generally, the elution of steroids is first performed with S1 for all the classes of steroids. Interestingly, with S1 and except for 15α-hydroxyoestriol, the retention volume of all the other compounds does not exceed 20 ml and the elution volume of each steroid lies between 1 ml (4-androstene-3,17-dione) and 4 ml (estriol).With estrogens and steroids that have retention volumes greater than 15 ml (6β-hydroxycortisol), the use of S2 significantly reduces their elution volumes without affecting their separation.

It was also shown that, with certain pairs of steroids that are incompletely separated with S1 and S2 (*e.g.*, 4-androstene-3,17-dione and testosterone, and 16α-hydroxyprogesterone and 17-hydroxyprogesterone), the use of S3 enables their separation to be carried out.

Whatever the selected system may be, it is not necessary to change the polarity during the chromatography, which makes the method simple and reproducible.

In addition to these results, we verified using plasma extracts (corresponding to 0.1–0.5 ml of plasma) loaded with steroid hormones (oestradiol, testosterone, progesterone, deoxycorticosterone and cortisol) that the elution volumes and chromatographic yields remained unchanged. In addition, preliminary results of radioimmunoassay measurements of plasma levels of estradiol, testosterone, progesterone, 17-hydroxyprogesterone and cortisol determined after the LH-20 chromatographic separation described here showed it to be reliable.

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