Journal of Chromatography, 193 (1980) 322–328

CHROM. 12,676

Note

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

H. GIPS*, K. KORTE, B. MEINECKE and P. BAILER

Zentrum für Frauenheilkunde und Geburtshilfe (gf. Direktor: Prof. Dr. W. Künzel) and Ambulatorische und Geburtshilfliche Veterinärklinik (gf. Direktor: Prof. Dr. H. Tillmann) der Justus-Liebig-Universität Giessen, 6300 Giessen (G.F.R.)

(First received November 12th, 1979; revised manuscript received January 11th, 1980)

Sephadex LH-20[1] and Celite microcolumns are commonly used for the separation of steroids. The preparation and packing of Celite columns is a more time consuming procedure than pipetting a slurry of Sephadex LH-20 into the columns. However, the systems described for separation of steroids on Sephadex LH-20[2–9] sometimes require large volumes of solvents and the flow-rates are low. Therefore, we have developed a microcolumn method of chromatography on Sephadex LH-20 by analogy with the Celite column chromatography of Abraham[10] with variable polarity of the eluent. In addition, time is saved by using pressure with solvent mixtures of low polarity.

EXPERIMENTAL

Materials

All organic solvents were purchased from Merck (Darmstadt, G.F.R.) ^{3}H-labelled steroids were obtained from NEN (Dreieichenhain, G.F.R.). Quickszint-Scintillator is a product of Zinsser (Frankfurt, G.F.R.) and Sephadex LH-20 a product of Pharmacia (Frankfurt, G.F.R.). Round fibre-glass plates (1.0 cm diameter) were obtained from Sartorius (Göttingen, G.F.R.). Glass columns (40 cm length and 0.8 cm I.D., widened to 1.0 cm I.D. at the bottom) were produced by a local glassblower according to our specifications.

Methods

Sephadex LH-20 was swollen overnight or for at least 4 h at room temperature in toluene–methanol (9:1). Two fibre-glass plates were placed at the bottom of each column. After vigorous shaking, 8 ml of a slurry containing 1.4 g of Sephadex LH-20 in toluene–methanol (9:1) were pipetted into each column. After the solvent mixture had run through the columns, the Sephadex was allowed to settle for 1 h. Subsequently, each column was equilibrated with 30 ml of the starting eluent. Then a fibre-glass plate was placed on top of the columns. The ethanolic solutions of ^{3}H-labelled steroids ($150 \cdot 10^3$–$250 \cdot 10^3$ cpm) were evaporated to dryness and the

* To whom correspondence should be addressed.

dried residue was dissolved in 0.3 ml of *n*-hexane–ethyl acetate–methanol (HEM) as described below. After application of this solution to the top of the columns, the sample vial was again rinsed with 0.3 ml of the solvent mixture and the rinsings were also added to the column.

The steroids were eluted with mixtures of HEM in which the amount of methanol remained constant but the ratio of *n*-hexane to ethyl acetate varied according the polarity of the steroids to be eluted. For determination of the elution patterns 1-ml fractions were collected and, after the addition of 10 ml of a scintillator, their radioactivity was determined in a beta counter (Isocap 300, Searle) with a counting efficiency of 38%. A low pressure of nitrogen (200 mmH_2O) was delivered by a manifold during equilibration and elution with solvent mixtures of low polarity. Care must to be taken to stop the pressure in time, otherwise the columns will run dry and have to be discarded.

The flow-rates of the various eluents are given in Table I.

TABLE I

FLOW-RATES OF THE DIFFERENT ELUTION MIXTURES

For elution mixtures of low polarity a nitrogen pressure of 200 mmH_2O is used to produce a practicable flow-rate.

HEM mixture*	*Flow-rate (ml/h)*	
	Without pressure	*With pressure*
20:0:1	—	23.5
19:1:1	—	30.6
18:2:1	64.2	—
17:3:1	67.5	—
16:4:1	76.3	—
14:6:1	75.3	—
8:12:1	67.6	—
0:20:1	38.0	—

* *n*-Hexane–ethyl acetate–methanol.

RESULTS

Chromatography of C_{21}-steroids

Progestins. Equilibration was carried out with 30 ml of HM (20:1), the solvent for the steroids was 2 × 0.3 ml of HM (20:1) and the eluents were HM (20:1) and HEM (16:4:1). The elution profile, volumes and eluent changes are shown in Fig. 1.

The recoveries* were as follows: 20α-dihydroprogesterone [HM (20:1), 5–10 ml], 78.5 ± 2.2%; progesterone [HM (20:1), 11–15 ml], 80.5 ± 1.9%; pregnenolone [HM (20:1), 16–22 ml] 87.9 ± 2.6% and 17α-hydroxyprogesterone [HEM (16:4:1), 1–8 ml), 82.2 ± 2.3%.

Corticoids. Equilibration was carried out with 30 ml of HEM (18:2:1), the solvent for the steroids was 2 × 0.3 ml of HEM (18:2:1) and the eluents were HEM

* Recoveries ± standard deviations were calculated from the means from five columns running simultaneously under identical conditions.

(18:2:1, 16:4:1, 14:6:1 and 0:20:1). The elution profile, volumes and eluent changes are shown in Fig. 2.

The recoveries were as follows: deoxycorticosterone (DOC) [HEM (18:2:1), 8–24 ml], 92.6 ± 1.3%; 17 α-hydroxyprogesterone [HEM (18:2:1), 8–24 ml], 91.1 ± 1.9%; 11-deoxycortisol [HEM (16:4:1), 1–13 ml], 78.6 ± 2.3%; cortisone [HEM (14:6:1), 1–10 ml], 77.5 ± 1.7%; and cortisol [HEM (0:20:1), 1–8 ml], 74.7 ± 2.6%.

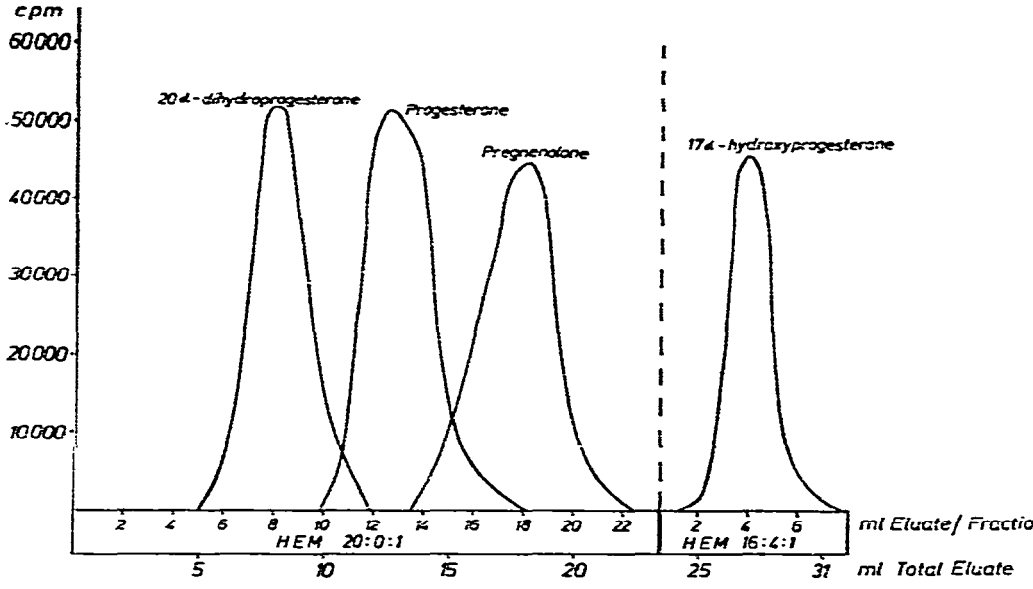

Fig. 1. Elution patterns of progestins.

Chromatography of C_{19}-steroids

Androstenedione and dehydroepiandrosterone (DHEA). Equilibration was carried out with 30 ml of HM (20:1), the solvent for the steroids was 2 × 0.3 ml of HEM (18:2:1) and the eluents were HEM (20:0:1 and 18:2:1). The elution profile volumes and eluent changes are shown in Fig. 3.

The recoveries were as follows: androstenedione [HEM (20:0:1), 13–27 ml], 82.1 ± 2.2%; and DHEA [HEM (18:2:1), 1–6 ml], 67.7 ± 2.5%.

5α-Dihydrotestosterone and testosterone. Equilibration was carried out with 30 ml of HM (20:1), the solvent for the steroids was 2 × 0.3 ml of HEM (18:2:1) and the eluents were HEM (20:0:1 and 16:4:1). The elution profile, volumes and eluent changes are shown in Fig. 4.

The recoveries were as follows: 5α-dihydrotestosterone [HEM (20:0:1), 13–29 ml], 90.3 ± 2.1%; and testosterone [HEM (16:4:1), 1–6 ml], 84.6 ± 3.0%.

Chromatography of C_{18}-steroids (estrone, estradiol-17β and estriol)

Equilibration was carried out with 30 ml of HEM (16:4:1), the solvent for the steroids was 2 × 0.3 ml of HEM (18:2:1) and the eluents were HEM (16:4:1,

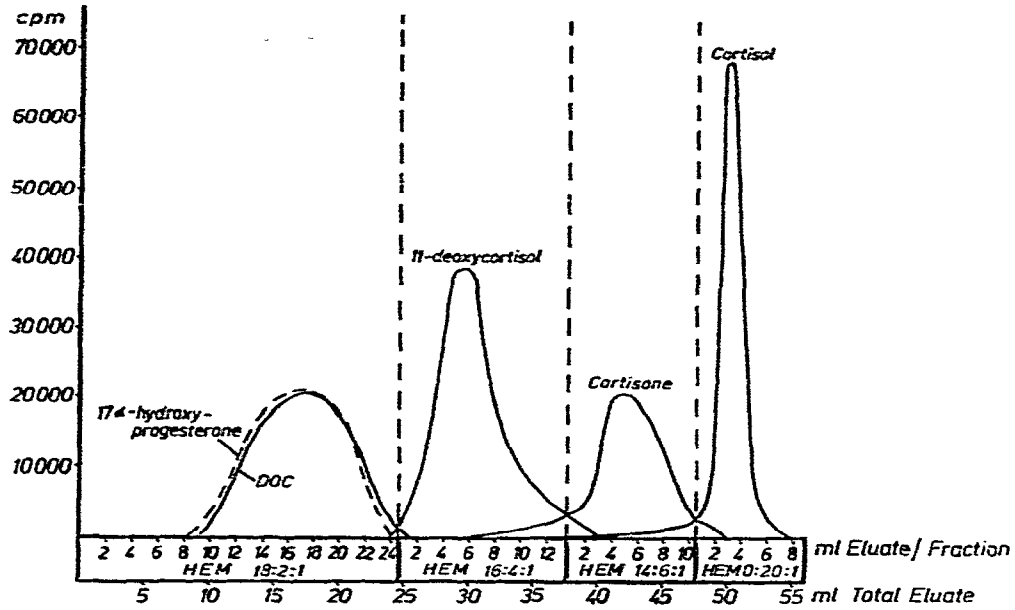

Fig. 2. Elution patterns of corticoids.

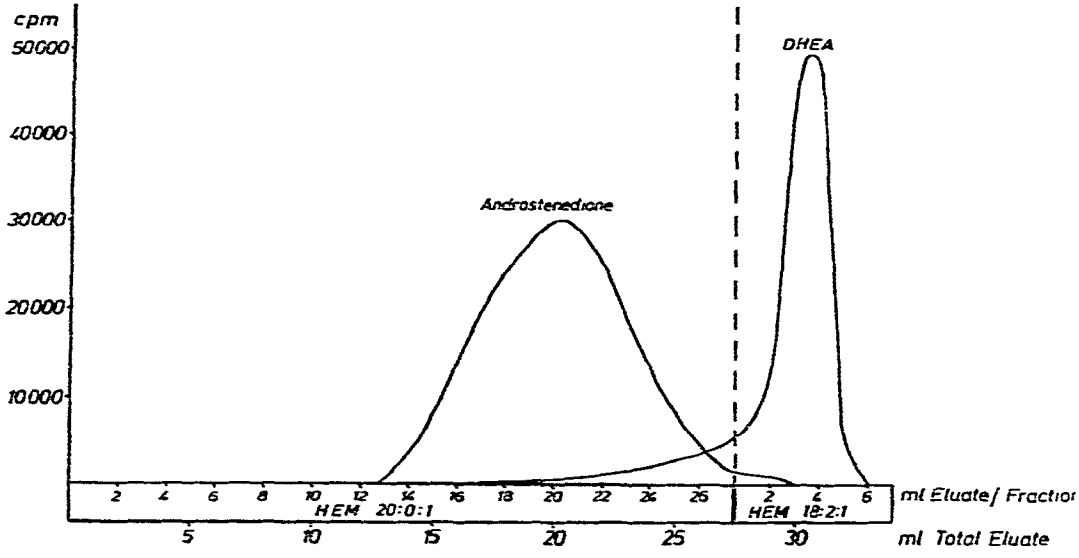

Fig. 3. Elution patterns of androstenedione and dehydroepiandrosterone (DHEA).

8:12:1 and 0:20:1). The elution profile, volumes and eluent changes are shown in Fig. 5.

The recoveries were as follows: estrone [HEM (16:4:1), 1–18 ml], 93.2 ± 2.3%; estradiol-17β [HEM (8:12:1), 1–7 ml], 76.7 ± 1.9%; and estriol [HEM (0:20:1), 1–15 ml], 84.6 ± 2.7%.

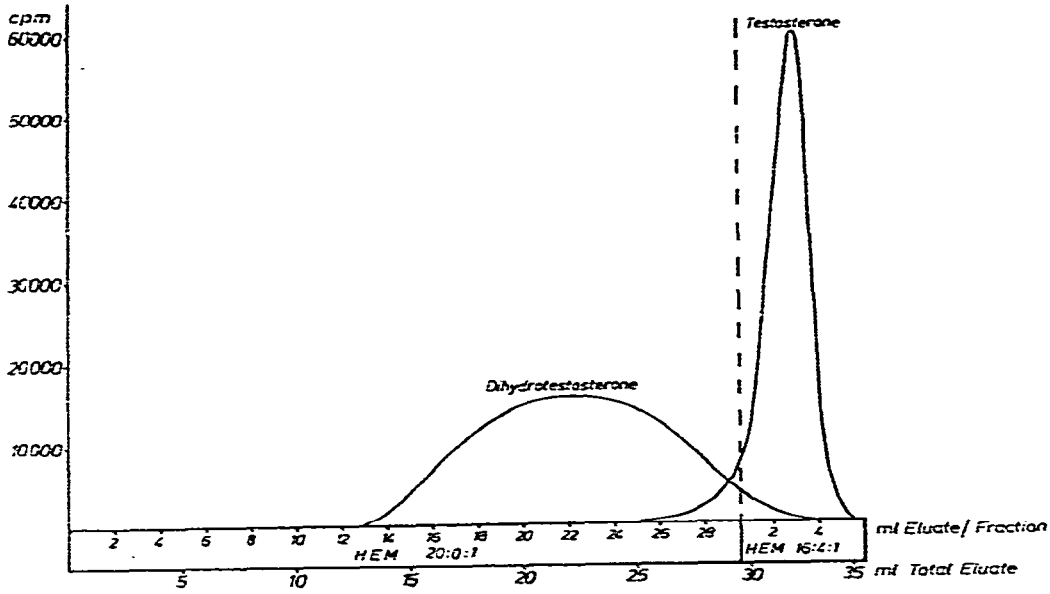

Fig. 4. Elution patterns of testosterone and 5α-dihydrotestosterone.

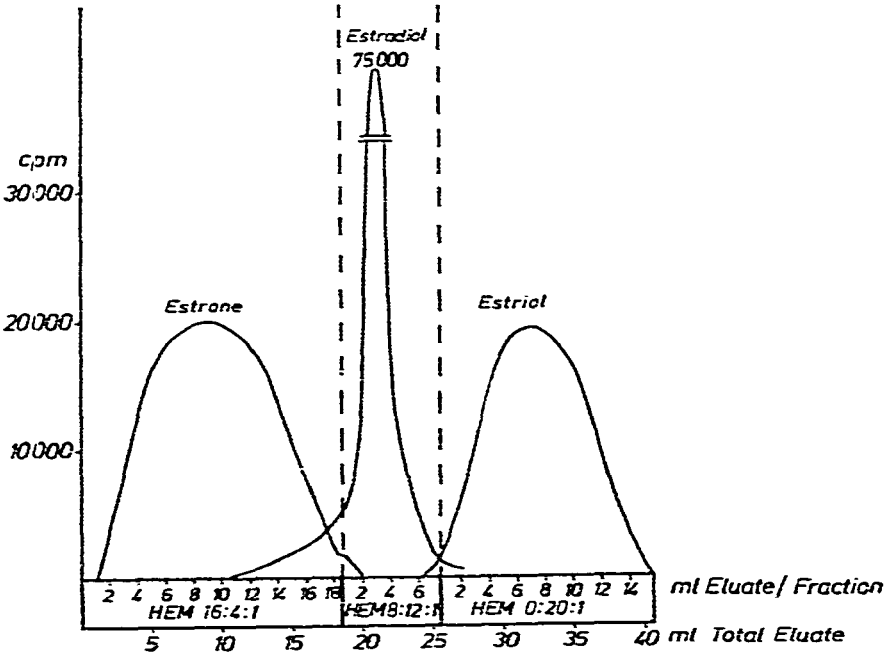

Fig. 5. Elution patterns of estrogens.

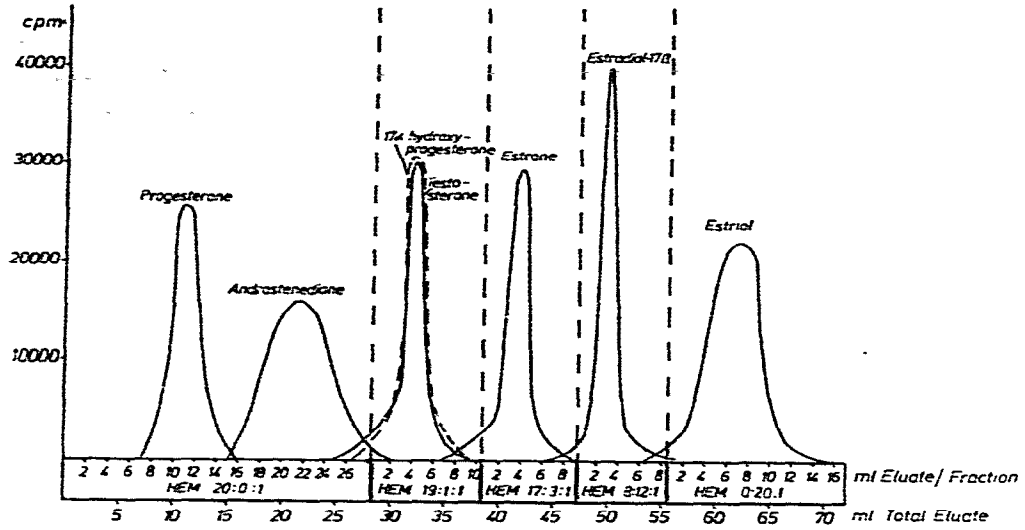

Fig. 6. Elution patterns of progestins, androgens and estrogens.

Chromatography of progestins, androgens and estrogens

Equilibration was carried out with 30 ml of HM (20:1), the solvent for the steroids was 2 × 0.3 ml of HEM (19:1:1) and the eluents were HEM (20:0:1, 19:1:1, 17:3:1, 8:12:1 and 0:20:1). The elution profile, volumes and eluent changes are shown in Fig. 6.

The recoveries were as follows: progesterone [HEM (20:0:1), 7–14 ml], 93.0 ± 1.8%; androstenedione [HEM (20:0:1), 16–27 ml], 87.4 ± 2.3%; testosterone [HEM (19:1:1), 1–9 ml], 84.6 ± 1.9%; 17α-hydroxyprogesterone [HEM (19:1:1), 1–9 ml], 82.9 ± 2.1%; estrone [HEM (17:3:1), 1–8 ml], 77.8 ± 2.2%; estradiol-17β [HEM (8:12:1), 1–8 ml], 73.6 ± 3.2%; and estriol [HEM (0:20:1), 1–14 ml], 63.1 ± 3.4%.

DISCUSSION

Whereas the packing of Celite columns is very tedious and time consuming, requiring experience to reproduce the same flow-rate from column to column, the packing of columns with Sephadex LH-20 by pouring it into the column as a slurry in an organic solvent does not require great skill and is faster. To save time and organic solvent we developed our Sephadex LH-20 microcolumn chromatography by changing the polarity of the eluents and applying a low pressure of nitrogen. A greater polarity of the eluents is achieved by increasing the ratio of ethyl acetate to *n*-hexane. The flow-rate in Sephadex LH-20 columns depends on the polarity of the eluent. Low flow-rates are encountered with eluents of low polarity, *e.g.*, HM (20:1). Therefore, a low pressure of nitrogen (200 mmH_2O) is used for eluents with low polarity. In pilot studies we found that the elution profile is not changed by this low pressure, but if it exceeds 300 mmH_2O the elution profile will change, the separation will be less sharp and the reproducibility will suffer. Elution with eluents of high polarity requires no pressure.

ACKNOWLEDGEMENTS

The technical assistance of I. Dennstedt, S. Reising and M. Scholz is gratefully acknowledged. The authors thank Mrs. Rosemarie Achenbach for drawing the figures.

REFERENCES

1 *Sephadex LH-20 Chromatography in Organic Solvents*, Pharmacia, Uppsala, Sweden, 1977.
2 B. R. Carr, G. Mikhail and G. L. Flickinger, *J. Clin. Endrocrinol.*, 33 (1971) 558.
3 H. W. Chung and L. E. Lundy, *Steroids*, 18 (1971) 91.
4 T. Ito, I. Woo, R. Haning and R. Horton, *J. Clin. Endocrinol*, 34 (1972) 106.
5 G. Mikhail, H. W. Chung, M. Ferin and R. L. Vande Wiele, in F. G. Peron, B. V. Caldwell (Editors), *Immunologic Methods in Steroid Determination*, Appleton-Century-Crofts, New York, 1970, p. 113.
6 G. Mikhail, C. H. Wu, M. Ferin and R. L. Vande Wiele, *Steroids*, 15 (1970) 333.
7 B. E. P. Murphy, *Nature New Biol.*, 232 (1971) 21.
8 J. E. Powell and V. C. Stevens, *Clin. Chem.*, 19 (1973) 210.
9 E. Youssefnejadian, E. Florensa, W. P. Collings and I. F. Sommerville, *Steroids*, 20 (1972) 773.
10 G. E. Abraham, *Acta Endocrinol. (Copenhagen), Suppl.*, 183 (1974) 7.