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Note

Separation of 5α-pregnan-3,20-dione and progesterone on Sephadex LH-20

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Previous methods for the measurement of 5α-pregnan-3,20-dione (5α-DHP) have relied on gas–liquid chromatography following an initial separation by thin-layer chromatography[1–3]. More recent methods for the assay of 5α-DHP, including the competitive protein binding method developed in this laboratory[4] as well as the use of radioimmunoassays[5] require a method of separation of 5α-DHP from other steroids which cross-react in the assay but also does not invalidate the assay due to blank values. Studies in this laboratory indicated a prohibitive blank value with the use of thin-layer chromatographic separations of 5α-DHP. Therefore, separation on Sephadex LH-20 (Pharmacia, Piscataway, N.J., U.S.A.) was developed because of the minimal interference in blank values with radioligand assays. This method represents a modification of the procedure of Labhsetwar and Watson[6] for the purification of progesterone on Sephadex LH-20.

EXPERIMENTAL

Sephadex LH-20 (500 mg) was swelled overnight in excess 90% aqueous methanol in individual covered vessels (liquid scintillation vials). A 5-ml disposable pipet (Corning, No. 7079) was used as a column with glass beads as support. The entire Sephadex slurry was added to the column and allowed to settle. This resulted in a column height of 7.5 cm. The column was then washed with 15 ml of 90% methanol, followed by 15 ml of the eluent (isooctane saturated with 90% methanol). Samples were then applied to the column in 0.2 ml of the eluent followed by a second application of the rinsed vessel. Steroids were eluted with further additions of the eluent which was collected in 1-ml fractions. Separations were done at room temperature and atmospheric pressure. Tritiated or ^{14}C-labelled steroids were added to the column either individually or two at a time for dual-labelled separation and included [^{3}H]-5α-DHP, [^{3}H]- or [^{14}C]-progesterone, [^{14}C]-17α-hydroxyprogesterone (17α-OHP), [^{3}H]-21-hydroxypregn-4-ene-3,20-dione (DOC) or [^{3}H]-pregnenolone.

RESULTS

Characteristic patterns of recovery for steroids were identical for either single-labelled or dual-labelled steroid separations. Fractions of 1 ml were collected and counted in a liquid scintillation spectrometer. 5α-DHP was recovered in fractions 4

TABLE I

ELUTION PATTERN OF 5α-DHP AND PROGESTERONE FROM SEPHADEX LH-20 COLUMNS

Each fraction was 1 ml; recovery is the mean ± S.E.M.

5α-Pregnan-3,20-dione (n = 14)		*Progesterone (n = 4)*	
Fraction no.	*% Recovery*	*Fraction no.*	*% Recovery*
1	1.8 ± 0.3	10	6.6 ± 0.9
2	0.6 ± 0.1	11	34.4 ± 0.9
3	1.7 ± 0.3	12	35.6 ± 2.2
4	47.5 ± 2.4	13	9.5 ± 1.2
5	28.4 ± 2.5		
6	4.7 ± 0.6		
7	1.5 ± 0.1		
8	0.4 ± 0.1		

and 5 with a mean recovery of 75.9 ± 3.8% ($n = 14$) for the 2 ml collected. Progesterone was recovered in fractions 11 and 12 with a 70% recovery ($n = 4$) with no appreciable amounts of progesterone recovered before 10 ml of eluent. Percentage recoveries for 5α-DHP and progesterone are presented in Table I. DOC, pregnenolone and 17α-OHP were not eluted from the column when as much as 15 ml of eluate was used. Both 5α- and 5β-DHP are eluted from the LH-20 column in the same fraction, with the greatest recovery of each in the fifth 1-ml fraction. Because of the minimal binding of 5β-DHP with the progesterone binding protein of pregnant guinea pigs[7,8], and the expected low levels in biological samples, no further effort was made to separate these two isomers.

Sephadex LH-20 column chromatography, using 90% aqueous methanol as the stationary phase and isooctane (saturated with 90% methanol) as the mobile phase, has been developed in this laboratory for the separation of 5α-DHP and progesterone in serum extracts. This method is rapid, economical and gives a low blank value in the competitive protein binding assay of 5α-DHP.

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