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SEPHADEX LH-20 MULTIPLE-COLUMN CHROMATOGRAPHY FOR THE SIMULTANEOUS SEPARATION OF PROGESTERONE, DEOXYCORTICOSTERONE AND 17α-HYDROXYPROGESTERONE FROM SMALL PLASMA SAMPLES

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SUMMARY

A simple and convenient chromatographic method is described for the simultaneous and complete separation of the unconjugated steroids progesterone, deoxycorticosterone and 17α-hydroxyprogesterone on 40-cm columns of Sephadex LH-20 using a water-saturated solvent system containing *n*-heptane–chloroform (1:1) plus 0.25% of ethanol. Manual operation of up to ten columns run in parallel is facilitated by the use of graduated, cylindrical, solvent reservoirs on top of the columns. Thus, negligible column-to-column, and only limited day-to-day, variations occur in the elution patterns, and constant high recoveries are obtained. When combined with a previously described 60-cm LH-20 chromatography, eight important corticosteroids can be isolated individually from 16 to 20 small plasma samples per day.

INTRODUCTION

Chromatographic separation of progesterone (P), deoxycorticosterone (DOC) and 17α-hydroxyprogesterone (17α) individually has been reported by several workers[1–6]. The simultaneous separation of these unconjugated steroids from plasma extracts, however, has not been attempted as often[7–9]. In a previous report[10], complete resolution of P, DOC and 17α was not obtained on a 60-cm column of Sephadex LH-20 using the solvent system methylene chloride–methanol (49:1), as had been suggested by Murphy[7]. Since we are interested in the determination of each of these corticosteroids which represent precursors of either the mineralocorticoid or glucocorticoid biosynthetic paths, we have chosen a second chromatographic system for a subsequent separation of these steroids prior to radioimmunological determination. A convenient solvent system for the separation of progesterone, 20α-dihydroprogesterone and 17α-hydroxyprogesterone has also been reported by Murphy[7]. This system can also be used to elute DOC instead of 20α-dihydroprogesterone. In combination with our automated 60-cm LH-20 multiple-column chromatography[10], the system allows the simultaneous separation of eight individual corticosteroids (P, DOC, 17α,

corticosterone, 11-deoxycortisol, aldosterone, cortisone and cortisol) as pure fractions from the same small plasma sample.

EXPERIMENTAL

Materials

Borosilicate-glass columns (44 cm × 11 mm I.D.) were made with a ground-in connector at the top (NS 14/23) and a frit having pores of 40–90-μm width (Schott, Mainz, G.F.R.) at the bottom. Below the frit, a PTFE stopcock (1.5 mm, NS 12.5) having a 3-cm mouthpiece was attached. Solvent reservoirs were made out of 100-ml borosilicate-glass calibrated cylinders, the bottom of which had been replaced by a funnel-like mouthpiece with a ground connector (NS 14/23) which fitted the tops of the columns. Glass tubes (16 × 100 mm) for collecting the eluate, and all other glassware used, were made steroid-free by previous heating to 500° (ref. 11). Miniature liquid scintillation vials were obtained from Zinsser, Frankfurt, G.F.R. Standard toluene or Bray's solution were used as scintillation fluids. Radioactivity was counted in a liquid scintillation spectrometer (Isocap 300, Searly Nuclear-Chicago Div.) with an efficiency of 65% for tritium. Analytical-reagent grade solvents (*n*-heptane, chloroform, ethanol, etc.) were purchased from Merck, Darmstadt, G.F.R., and used without further purification. Sephadex LH-20 was obtained from Pharmacia, Uppsala, Sweden. Tritiated steroids with a specific radioactivity of 40–60 Ci/mmole were obtained from New England Nuclear, Boston, Mass., U.S.A. *Ca.* $3 \cdot 10^5$–$5 \cdot 10^6$ cpm of each steroid, *i.e.*, the amount to be used in a 2-month period, were purified by chromatography on a 40-cm column of Sephadex LH-20 using methylene chloride–methanol (49:1) as solvent.

Preparation of solvent and columns

The solvent system used here was prepared from 500 ml of *n*-heptane, 500 ml of chloroform, 2.5 ml of ethanol and *ca.* 5 ml of distilled sterile water which were mixed by twice thoroughly shaking and separating the mixture using a 2-l separating funnel. Special care was taken in order to remove all of the water droplets. Sephadex LH-20 (10.0 g per column) was allowed to swell overnight at 4° in about three volumes of the solvent system. Since this solvent has a lower density than the gel, thorough mixing is essential at the beginning of the swelling period, as well as prior to the packing of the columns. The rinsed columns, each containing *ca.* 5 ml of the solvent, were packed by pouring small amounts of the LH-20 sludge into the columns using steroid-free disposable Pasteur pipettes. With the stopcocks permanently open, the gel was allowed to settle by gravity. All of the columns were packed at the same time; a gel height of 37 cm was reached after 2 h. After packing, all of the columns were rinsed with one column volume (35 ml) of the solvent, and were stoppered and left overnight at room temperature.

Running of the columns

In order to obtain sharp steroid peaks, the extract was layered very carefully on to the almost dry top of the gel. The extract had been previously dissolved in 250 μl of the solvent, and was quantitatively transferred by means of 1-ml tuberculin glass syringes followed by a washing with the same solvent volume. With the

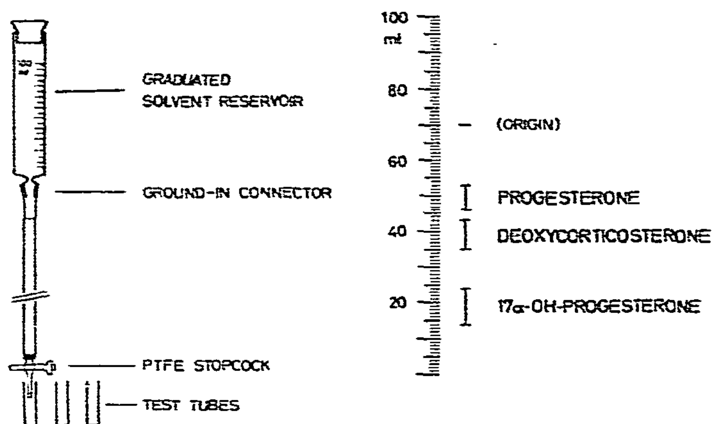

Fig. 1. Diagram of the 40-cm chromatographic column with graduated solvent reservoir, and calibration showing the collection limits for the three steroids to be isolated.

stopcocks open, the transferred volume of 500 μl sank completely into the gel in *ca.* 15 sec. The solvent was then carefully layered in small portions on to the dry surface of the gel, thereby avoiding any major turbulence. When the supernatant solvent had reached the top ground-in connector, the reservoir was attached and then rapidly filled with solvent to the 70-ml mark. The chromatographic column and solvent reservoir and its calibration is outlined in Fig. 1. The reservoir could easily be filled in 30 sec, during which time less than 1 ml of solvent were eluted. By charging one column after another, eight columns were usually eluted together. The use of graduated, cylindrical, solvent reservoirs on the top of the column greatly facilitated the manual volume fractionation of the eluate. Appropriate eluate fractions, as determined by the actual solvent level in the reservoir, were collected in ordinary test-tubes.

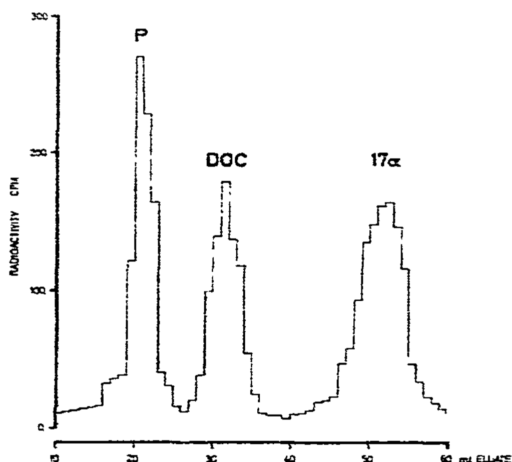

Fig. 2. Elution pattern of tritiated steroids progesterone (P), deoxycorticosterone (DOC) and 17α-hydroxyprogesterone (17α) on 40-cm columns of Sephadex LH-20 using a water-saturated solvent system of *n*-heptane–chloroform (1:1) plus 0.25% of ethanol.

TABLE I

ELUTION VOLUMES AND COLLECTION LIMITS OF PLASMA CORTICOSTEROIDS ISOLATED ON 40-cm COLUMNS OF SEPHADEX LH-20

Steroid	*Elution volume (ml)*	*Fractions collected (ml)*	*Limits marked on reservoir (ml)*	*Fraction pool volume (ml)*
Progesterone	20	17–24	53–46	7
Deoxycorticosterone	32	27–35	43–35	8
17α-Hydroxyprogesterone	53	46–56	24–14	10

For localization experiments, 1-ml fractions were collected directly in miniature scintillation vials.

RESULTS

Localization of the steroid peaks

Fig. 2 shows a typical elution pattern of a mixture of the tritiated steroids P, DOC and 17α (each containing *ca.* 1500 cpm), obtained by chromatography on a 40-cm column of Sephadex LH-20 using a water-saturated solvent system composed of *n*-heptane–chloroform (1:1) plus 0.25% of ethanol. The peaks obtained are rather sharp and almost symmetrical. In the two portions of the chromatogram between the three peaks, as well as before and after the peaks, only background level activity could be detected. Pertinent chromatographic data, including elution volumes, collected fractions and their limits as marked on the calibrated reservoir (Fig. 1), originating from a starting volume of 70 ml, are listed in Table I.

Recoveries

When tritiated P, DOC and 17α were eluted separately during localization experiments, average "analytical" recoveries of radioactivity collected within the limits shown in Table I were 86.1, 84.4 and 80.6%, respectively. In general, slightly decreasing recoveries were observed with increasing elution volumes. During routine use, on the other hand, a fraction pool from the preceding automated LH-20 chromatography on 60-cm columns[10] containing the unseparated fractions of P, DOC and 17α was chromatographed. These "preparative" recoveries were considerably lower

TABLE II

RECOVERIES OF TRITIATED PLASMA CORTICOSTEROIDS AFTER EXTRACTION AND TWO SUBSEQUENT CHROMATOGRAPHIES ON 60-cm AND 40-cm COLUMNS OF SEPHADEX LH-20

Steroid	*Radioactivity recovered ±S.D. (%)*	*Number of 40-cm columns eluted*	*Mean inter-column coefficient of variation (%)*
Progesterone	55.9 ± 12.1	144	11.6
Deoxycorticosterone	51.5 ± 10.2	144	7.6
17α-Hydroxyprogesterone	44.3 ± 9.3	144	6.7

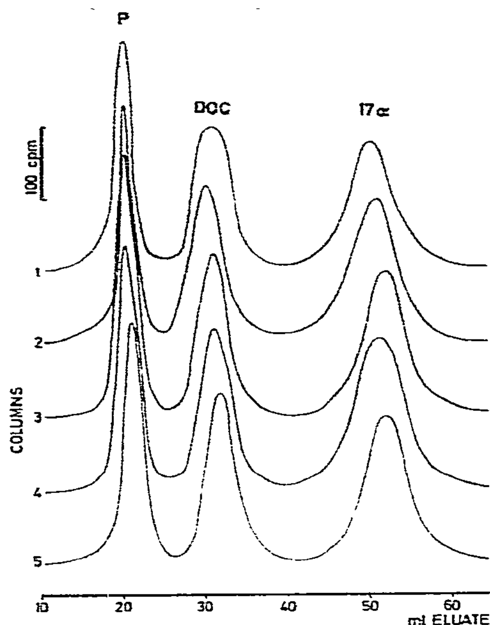

Fig. 3. Inter-column variation of elution patterns of the tritiated steroids one day after the packing of the columns.

therefore, as shown in Table II, since they included procedural losses of tracer steroids from plasma extraction (of *ca.* 10%) and two subsequent chromatographies. Nevertheless, the steroid amounts which were recovered were sufficient for sensitive radioimmunological quantitation.

Reproducibility

The elution profiles from five columns run in parallel 1 day after the packing of the columns are shown in Fig. 3. It can be seen that both the location and the width of the steroid peaks eluted from these columns are almost identical. This parallelism of elution patterns between the different columns remained unaltered even after 15 runs. Due to an initial shrinking of the gel column of *ca.* 2 cm after six elutions, the steroid peaks shifted slightly towards lower elution volumes, as shown in Fig. 4. Since this shifting was uniform in all of the columns, all three steroids were collected 2 to 3 ml earlier, as soon as the recoveries were found to be slightly decreasing. This usually occurred after six to eight elutions.

Practicability

In general, six to eight columns were eluted together. It was not necessary to start all of the columns at the same time, since the relatively simple elution pattern did not require automatic fraction collection. It was most advantageous to run a total of eight columns with a column-to-column phase difference which varied between 1 and 3 min. One technician can easily elute eight columns two or three times during one working day, including rinsing of the columns with *ca.* 35 ml of solvent after each run. After about 15 elutions, all of the columns were emptied (which was

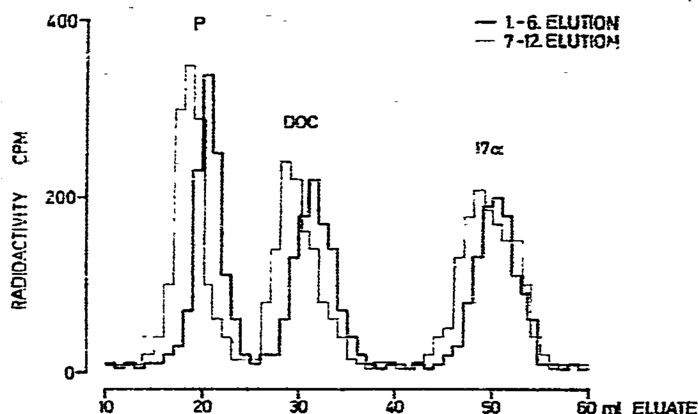

Fig. 4. Shifting of the elution patterns of the tritiated steroids towards 2–3 ml lower elution volumes after six runs.

greatly facilitated by applying compressed air to the bottom mouthpiece of the column) and re-packed with Sephadex, which was either fresh or recycled after purification[12].

DISCUSSION

P and its metabolites from hydroxylation in either the mineralocorticoid or glucocorticoid biosynthetic paths, *i.e.*, DOC and 17α, respectively, can be separated completely and simultaneously from each other, despite their very similar polarity and molecular size, by use of a 40-cm column of Sephadex LH-20 and a solvent system suggested by Murphy[7]. The extension of this method to a multiple-column scale as described in the present study was greatly facilitated by the introduction of a calibrated solvent reservoir on the top of each column. The reservoir allows both simple consecutive charging and easy parallel elution of about eight to ten chromatographic columns. Sophisticated automation devices such as fraction collectors, control and programmer units, even tubing, connectors, etc. were unnecessary. Nevertheless, column-to-column variation was minimal and the recoveries of tracer steroids were very satisfactory. With a minor correction of the collection limits after about six runs, all of the columns could be eluted up to 15 times before being re-packed. There was almost no change in the flow-rates between columns which were run in parallel as well as during one elution (despite the decrease in hydrostatic pressure). A constant and slightly low room temperature (18–20°) also contributed to the good separation and reproducibility.

In our experience, the chromatographic system described here is not convenient for separating total plasma extracts without a preceding chromatography in order to eliminate unconjugated steroids which are more polar than 17α. Such corticosteroids, *e.g.*, cortisol, cortisone and even corticosterone, are rather firmly retained by the gel, resulting in excessively high elution volumes and in virtually indiscernible peaks. Plasma steroids of similar polarity and structure, however, such as androstenedione and testosterone, are eluted on these columns from 19 to 23 ml (together with P)

and from 50 to 59 ml (overlapping 17α), respectively. Fortunately, their chromatographic interference can be eliminated in the subsequent radioimmunoassays by the specificity of the antisera used which show negligible cross-reactions with androgenic steroids. In combination with an appropriate prior separation procedure, therefore, the present chromatographic system provides a useful tool for the isolation of the clinically and experimentally important corticosteroid hormones P, DOC and 17α from a single plasma sample prior to radioimmunological determination.

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