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AUTOMATION OF MULTIPLE SEPHADEX LH-20 COLUMN CHROMATOGRAPHY FOR THE SIMULTANEOUS SEPARATION OF PLASMA CORTICOSTEROIDS

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SUMMARY

An automated method of Sephadex LH-20 chromatography has been developed for the simultaneous and complete separation of corticosterone, 11-deoxycortisol, aldosterone, cortisone and cortisol from a small plasma sample prior to radioimmunoassay. Using a controlled elution flow-rate of 40 ml/h and automated programme tape-controlled collection of the eluates from six columns eluted simultaneously, excellent reproducibility of elution patterns with minimal day-to-day and column-to-column variations could be achieved. The method can be easily extended and permits the reliable, rapid and easy routine separation of individual steroids from a single plasma or tissue extract on a multi-column scale.

INTRODUCTION

Sephadex LH-20 column chromatography of steroid hormones has been shown to have substantial advantages over other separation techniques such as paper and thin-layer chromatography and solvent partition[1–4]. It is rapid, blanks are invariably low, recoveries are sufficiently high and, most important, there is excellent reproducibility and the possibility of autómation. These features are particularly useful in combination with sensitive steroid radioimmunoassay methods[5]. Furthermore, Sephadex LH-20 chromatography permits the separation and subsequent determination of a variety of steroid hormones from a single plasma sample[6–9] or tissue extract[10]. This is extremely convenient and economical whenever very little plasma is available, such as in paediatric and experimental endocrinology.

This study was designed to developed an automated Sephadex LH-20 multicolumn chromatographic system for the improved isolation of several major corticosteroids from single plasma samples.

EXPERIMENTAL

Materials

Sephadex LH-20 was obtained from Pharmacia, Uppsala, Sweden. Analytical-

reagent grade solvents (Merck, Darmstadt, G.F.R.) were used without further purification. Tritiated steroids having a specific radioactivity of 40–60 Ci/mmole were purchased from New England Nuclear Corp., Boston, Mass., U.S.A. About 2×10^6 cpm of each steroid had been purified, no longer than 3 months prior to use, by chromatography on a 40-cm Sephadex LH-20 column. Borosilicate glass columns (67.5 cm × 11 mm I.D.) were workshop-made according to our specifications with a ground-in connector at the top and a frit with 40–90-μm wide pores (Schott, Mainz, G.F.R.) at the bottom, serving as a gel support. Below it a capillary glass/PTFE valve (Rotaflo, Jobling Lab. Division, Stone, Staffs., Great Britain) with a simple bubble trap was attached. Glass capillary flow meters with a ruby float were obtained from Fischer & Porter, Göttingen, G.F.R. The fraction collector (Linear II), control unit (Type STZ), programmer, elution pump (Model N), PTFE and Viton tubing (0.5 mm I.D.) and couplings (Multifit) were purchased from Serva-Technik, Heidelberg, G.F.R.

Glass tubes (16 × 100 mm) for the collection of the eluate and all other glassware used were made steroid-free by previous heating to 500° (ref. 11). Normal-sized liquid scintillation vials and mini-vials (16 × 52 mm) were obtained from Packard, Frankfurt, G.F.R. Radioactive samples were counted in a Nuclear-Chicago Isocap 300 liquid scintillation spectrometer with an efficiency of 65% for tritium.

Column chromatography techniques

Preparation of columns. Sephadex LH-20 (15.0 g per 60-cm column) was allowed to swell overnight in the solvent system methylene chloride–methanol (98:2)[2]. By pouring the dilute Sephadex LH-20 sludge into the columns and allowing the gel to sediment by gravity with the valve at the bottom fully open, six columns were packed uniformly and simultaneously to a height of 60 cm in about 1 h. In our experience, placing a piece of PTFE gauze or a similar adaptor on top of the gel did not prevent floating of Sephadex particles in this solvent adequately. Therefore, no extra solvent was added to the top of the gel, and the columns were kept sealed by using a PTFE-fitted ground-glass stopper. By means of this precaution, the Sephadex gel did not become dry without addition of solvent over a period of about 2 weeks.

Procedure. During chromatography, solvent was pumped on top of the gel with a peristaltic elution pump feeding six columns. In order to prevent evaporation of the volatile solvent, the feeding tubes were fitted tightly through nylon stoppers on the tops of the columns. The pumping rate could be precisely adjusted to the elution flow-rate. A constant elution flow-rate during one chromatography as well as between different elutions is essential in order to obtain high reproducibility. Therefore, the elution flow-rate was regulated by a stopcock-like PTFE/glass capillary valve at the bottom of each column and was controlled by a very sensitive micro-flow meter behind it. As the flow meter can easily be disturbed by bubbles in the system, a simple small glass bubble-trap was placed between the valve and flow meter. This device was particularly useful whenever ambient temperatures rose above 20°, because increased bubble formation could then be observed behind the valve. The top of the flow meter was connected with the fraction collector by a short piece of PTFE capillary tubing, so the solvent volume between gel bed and the mouthpiece of the fraction collector was only 1.8 ml. As this small solvent volume extended over a total length of 1.75 m of capillary tubing (0.5 mm I.D.), virtually no re-mixing or even tailing of the separated corticosteroid peaks occurred.

Fractions of 1 ml were collected by a linear fraction collector, the multiple dispensing mouthpiece of which allows parallel collection of eluates from six columns running simultaneously. The fraction collector was volume-triggered by a glass siphon (1.0-ml delivery volume) within its photo-cell, which was attached to the end of the tubing from column No. 6, which was used as a control. In order to ensure a hydrostatic pressure necessary for an elution flow-rate of 40 ml/h, the fraction collector was placed in an ice-box about 1 m below the column rack.

For analytical purposes, *i.e.*, whenever the exact localization of radioactive steroid peaks had to be determined, the 1-ml fractions were each collected individually into a small scintillation vial containing 5 ml of toluene scintillator and fitting into the racks of the fraction collector. Recoveries of labelled steroids were counted with background subtraction and calculated by comparison with an adequate standard sample.

In general, whenever steroids were separated from plasma extracts prior to radioimmunoassay, the bulk of the 1-ml fractions of one steroid was pooled by the fraction collector in larger glass tubes. The exact sequence and position of these pools, as determined by previous localization experiments, was punched into the tape of a commercially available programmer unit controlling the fraction collector. The chromatographic set-up is outlined in Fig. 1.

A mixture of tritiated and unlabelled steroids, containing *ca.* 600 cpm of each steroid used, was transferred by use of 1-ml tuberculin glass syringes on to the columns

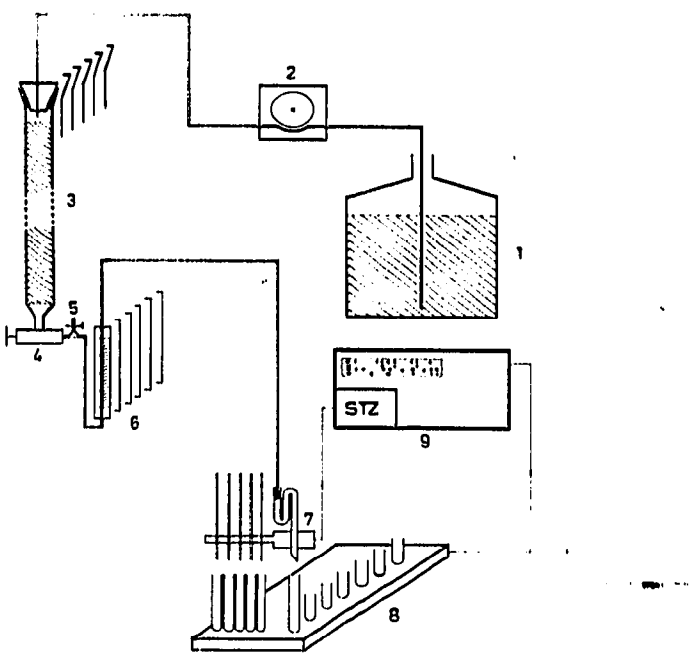

Fig. 1. Diagram of the automated multiple column chromatography system. 1, Solvent reservoir; 2, peristaltic elution pump; 3, Sephadex LH-20 columns; 4, PTFE/glass capillary valve; 5, bubble trap; 6, micro-flow meter; 7, 1-ml glass siphon with photo-cell; 8, linear fraction collector; 9, STZ control unit and punched tape-controlled programmer unit.

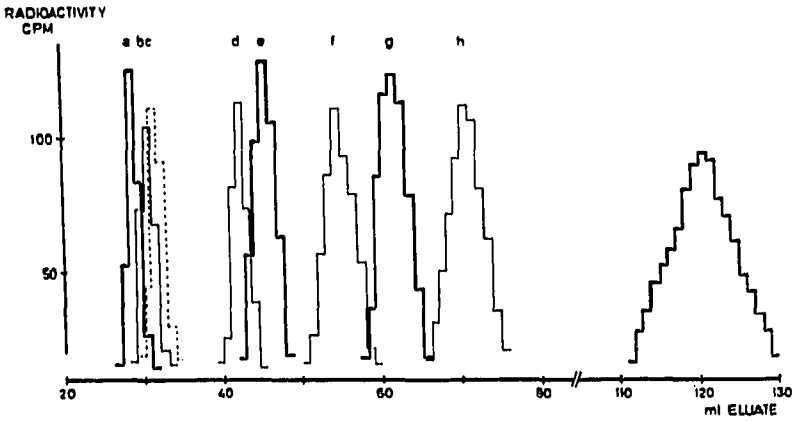

Fig. 2. Elution pattern of tritiated steroids on 60-cm Sephadex LH-20 columns using 40 ml/h of methylene chloride–methanol (98:2) as solvent. Peaks eluted: (a) progesterone; (b) deoxycorticosterone; (c) 17α-hydroxyprogesterone; (d) testosterone; (e) corticosterone; (f) 11-deoxycortisol; (g) aldosterone; (h) cortisone; (i) cortisol.

in a solvent volume of 0.250 ml with one subsequent washing with the same amount of solvent. The six columns could be charged consecutively within a period of less than 90 sec, which is the time required for the collection of one 1-ml fraction. After elution of the last steroid peak (cortisol), all columns were rinsed with at least one column volume (56 ml) of solvent. As measured on several occasions, no radioactivity could be detected during this period.

RESULTS

A typical elution pattern for nine tritiated steroids, obtained by chromato-

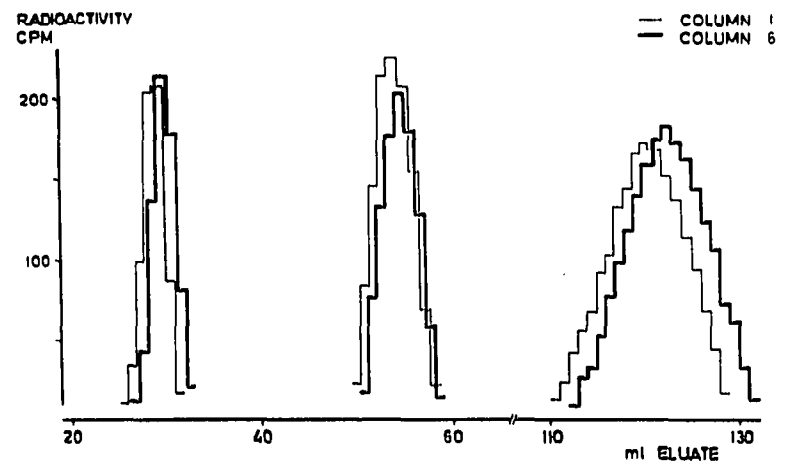

Fig. 3. Comparison of elution patterns of three tritiated steroids (progesterone, 11-deoxycortisol and cortisol) between columns 1 and 6, eluted simultaneously in automated multi-column chromatography.

graphy on a 60-cm Sephadex LH-20 column at a constant flow-rate of 40 ml/h, is shown in Fig. 2. With the solvent system 98:2 methylene chloride–methanol, complete separation of most physiologically important corticosteroids could be achieved. This is particularly useful for the separation of aldosterone from corticosterone, 11-deoxycortisol, cortisone and cortisol, whereas deoxycorticosterone could not be clearly separated from progesterone and its 17α-OH derivative on this column. Between testosterone and corticosterone, there was a slight overlap in up to two 1-ml fractions.

Even if relatively large amounts of steroid are chromatographed, the peaks do not become significantly wider. For example, when 40 ng of cortisone plus 400 pg of aldosterone were eluted together, there was only a slight (< 10%) overlap in one fraction between the two peaks.

In order to increase the capacity of the separation system, six columns were eluted together. In Fig. 3, the analytical chromatograms of three steroids (progesterone, 11-deoxycortisol and cortisol) eluted simultaneously on columns 1 and 6 are compared. These columns were the first and the last to be started. It can be seen that differences in the elution volumes do not exceed 1 and 2 ml in the 11-deoxycortisol and cortisol fractions, respectively, and therefore are negligible. Day-to-day variation of elution volumes was similarly low, not only on the same column but also on different columns, as shown in Fig. 4. Over a period of 10 months, both localization and width of the analyzed steroid peaks were virtually unaltered. All of these columns had been packed uniformly at the same time.

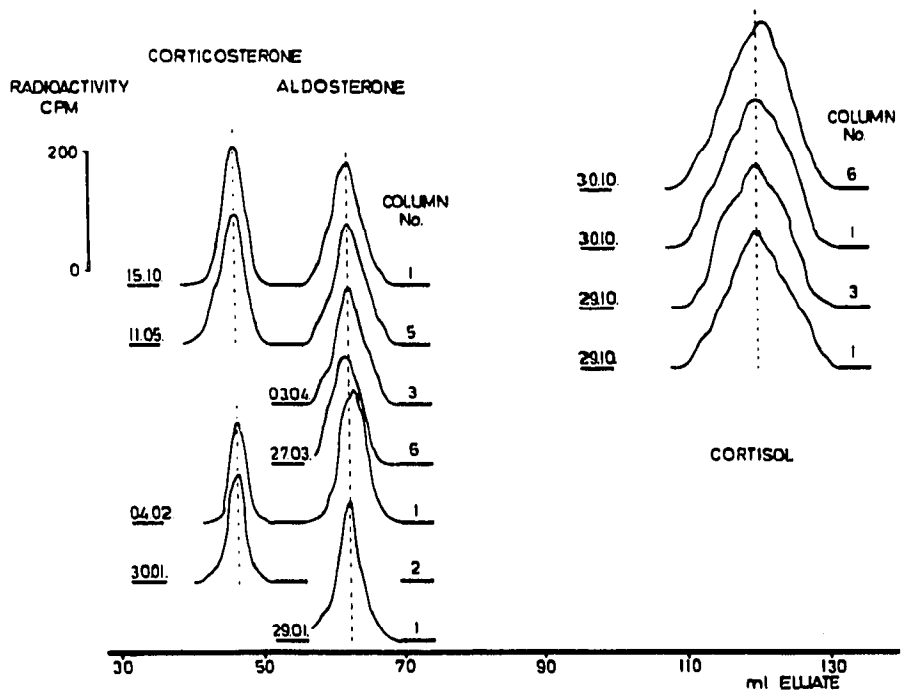

Fig. 4. Column-to-column and day-to-day variations of elution pattern of corticosterone, aldosterone and cortisol over a period of 10 months. All columns had been packed at the same time.

TABLE I

RECOVERIES OF TRITIATED STEROIDS ELUTED INDIVIDUALLY ON A 60-cm SEPHADEX LH-20 COLUMN IN 1-ml FRACTIONS

Steroid	*Radioactivity recovered (%)*	*Limits of fraction No.*
Progesterone	93.0	27– 30
Deoxycorticosterone	78.5	29– 32
17α-Hydroxyprogesterone	80.0	30– 33
Testosterone	90.0	40– 44
Corticosterone	81.0	43– 48
11-Deoxycortisol	78.0	50– 57
Aldosterone	78.0	57– 66
Cortisone	79.0	67– 75
Cortisol	75.0	112–129

With constant elution flow-rate and automated collection of the fractions, recoveries were usually high and reproducible. Table I gives the percentage of radioactivity recovered for nine tritiated steroids separated individually within the limits chosen. As expected, the recoveries decreased somewhat towards the end of the elution because of wider and less sharp steroid peaks.

In order to eliminate the possibility that the corticosteroids to be isolated from plasma extracts might be contaminated by surrounding steroid peaks, which might occur in various pathological conditions, these corticosteroids (*e.g.*, corticosterone, aldosterone and cortisol) were collected within relatively narrow limits. As a result, their mean recoveries decreased, but the reproducibility over a long period of time was high, as indicated by the low standard deviations (Table II). Similarly, on the basis of these percentage recoveries, the coefficients of variation between the six columns of the automated multi-column chromatography system were low, ranging between 6.7 and 10.3%.

The exact elution programme for the corticosteroids isolated routinely, according to which the programmer punched tape was prepared, is shown in Table III. The total elution time, including rinsing the columns with one column volume of solvent after collection of the cortisol peak, was 4½ h, *i.e.*, the columns could be eluted twice in one working day by one technician. In our present set-up, in which a standard steroid mixture is eluted on one of the six columns as an internal quality control, 30 corticosteroids can be isolated from 10 plasma samples per day.

TABLE II

RECOVERIES OF PLASMA CORTICOSTEROIDS IN AUTOMATED MULTI-COLUMN CHROMATOGRAPHY

Steroid	*Radioactivity recovered ±S.D. (%)*	*Number of columns eluted*	*Mean inter-column coefficient of variation (columns 1–6) (%)*
Corticosterone	66.2 ± 6.5	66	8.1
Aldosterone	63.9 ± 4.9	66	6.7
Cortisol	49.4 ± 5.9	66	10.3

TABLE III

ELUTION VOLUMES AND FRACTION LIMITS OF PLASMA CORTICOSTEROIDS ISOLATED AUTOMATICALLY ON SEPHADEX LH-20 COLUMNS

Elution volume (ml)	*Steroid*	*Fraction numbers collected*	*Fraction pool volume (ml)*
28	Progesterone		
30	Deoxycorticosterone		
31	17α-Hydroxyprogesterone		
42	Testosterone		
45	Corticosterone	44– 48	5
54	11-Deoxycortisol		
61	Aldosterone	58– 65	8
72	Cortisone		
120	Cortisol	114–125	12

DISCUSSION

Liquid gel column chromatography on Sephadex LH-20 with the solvent system of low polarity used in this work separates plasma corticosteroids according to their polarity[12,13]. The advantages of this technique over other chromatographic procedures such as paper and thin-layer chromatography are well established and have been described in detail by several workers[1–5]. Yet, the high density of the solvent containing 98% of methylene chloride makes it necessary to avoid any solvent supernatant on top of the gel in order to prevent it from floating in the solvent. Therefore, it is essential to feed the columns evenly with solvent at exactly the same rate as that of the elution flow. This can be accomplished easily by simple automation. Further, automation offers the considerable advantage of the controlled elution of a number of columns simultaneously. In the chromatographic set-up described here, minimal day-to-day and column-to-column variability of elution volumes and recoveries was achieved mainly by keeping the elution flow-rate always constant at 40 ml/h. Day-to-day variations were further reduced by maintaining a relatively constant temperature range (18–20°) in the laboratory[12]. Column-to-column variations, on the other hand, were minimized by the following precautions: (1) all columns to be eluted together had been packed uniformly at the same time; (2) steroid mixtures or dried plasma or tissue extracts dissolved in 0.250 ml of solvent were transferred consecutively very quickly on to the columns within no more than the time needed for the collection of one fraction (90 sec); and (3) volume-triggered automated collection of the eluate fractions of all columns was performed by the same fraction collector controlled by one elution programme tape.

Automated column chromatography on Sephadex LH-20 permits the accurate, reproducible, rapid and easy routine separation of a multitude of individual steroids from a single plasma sample on a large, multi-column scale. Using one linear fraction collector, up to ten columns can be eluted simultaneously at a controlled flow-rate. If more plasma extracts have to be chromatographed in the same period, several fraction collectors, each collecting from up to ten columns, could be controlled by the elution programme tape of one programmer.

The possibility of isolating a variety of important corticosteroid hormones from the same plasma sample or tissue extract often, in our opinion, serves many clinical and experimental purposes, whenever possible changes in plasma or tissue steroid patterns have to be investigated and/or very little material is available. Quantitation of the isolated steroids by sensitive radioimmunoassay procedures is facilitated by the low blanks and the relatively high recoveries of the method described here. Even corticosteroids that are not completely separated from other overlapping steroids (*e.g.*, corticosterone slightly contaminated with testosterone) can be assayed accurately provided that an antibody showing negligible cross-reaction with the contaminating steroid is used.

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