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AN AUTOMATED MULTICOLUMN SYSTEM FOR CHROMATOGRAPHY OF ALDOSTERONE ON SEPHADEX LH-20 IN WATER

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SUMMARY

A semi-automated method of Sephadex LH-20 column chromatography using water as a solvent is described. Simple modifications to an LKB fraction collector allowed simultaneous collection from ten columns whose flow rate was controlled using a multichannel peristaltic pump. Highly reproducible separations of aldosterone from a mixture of corticosteroids was obtained. Aldosterone was the first steroid eluted from the column and was followed immediately by the marker dye, isatin, which could be conveniently employed to locate this steroid. The system is simple and when used in conjunction with radioimmunoassay was blank-free. The separation profile was not altered by applying the samples to the columns in 2.0 ml of Krebs–Ringer Bicarbonate solution containing 4% bovine serum albumin and could therefore be used to process incubation media without prior solvent extraction.

INTRODUCTION

The measurement of aldosterone often requires some form of purification prior to radioimmunoassay. Non-chromatographic techniques have relied on the formation of the γ-lactone derivative [1–4] or purification using antibodies or binding proteins [5–7]. Oxidation of aldosterone is a lengthy procedure which may result in losses and the assay requires an antibody to aldosterone-γ-lactone. The immunological methods are also time consuming and there are problems with high blanks [5] and low recoveries [6].

A promising approach involves the use of highly specific antibodies. Those raised to aldosterone-3-oxime-BSA [8–16] have recently been used in direct assays of aldosterone in plasma without prior extraction or purification [14–16]. However, these highly specific antibodies are not yet generally available and chromatographic purification of plasma extracts is often still necessary before radioimmunoassay. In addition, chromatography is frequently required for the validation of steroid determinations which do not use chromatography [5, 6, 10, 12, 14, 16].

Column chromatography using Sephadex LH-20 has been widely used to separate corticosteroids [17–28] and it is possible that this system is superior to paper or thin layer chromatography because the gel is blank free and the columns are reusable, reproducible and less time consuming. The most common solvent system for these columns has been methylene chloride–methanol (98:2, V/V) [17–19, 21, 23, 26, 28] but the use of organic solvents requires special equipment such as solvent resistant glass columns, stopcocks and tubing. Sephadex LH-20 floats in methylene chloride making packing of the columns difficult and evaporation must be avoided to prevent the columns drying out, particularly if they are to be stored for long periods. The problem of the gel floating is avoided using the solvent system benzene-methanol (96:4, V/V) [25]. However benzene is toxic and the problems of solvent corrosion still apply in this system. A thin layer chromatography system on polyamide using methylene chloride-methanol (98:2, V/V) has all the advantages of Sephadex LH-20 chromatography without gel flotation, gel fissure and the necessity for large solvent volumes [26]. However equilibration of this solvent system is a problem and because of the soft polyamide, application of the sample is difficult and sample volume limited.

A further advantage of column chromatography over other methods of steroid separation is the possibility of automation. Automated systems using organic solvents have been described [23, 28] but special equipment is required for handling these solvents and automation is consequently difficult and expensive. We therefore investigated the possibility of using a water-based system [20, 24].

In this paper we describe a semi-automated method of separating aldosterone from some other corticosteroids using common, easily adapted laboratory equipment. Following the reported use of marker dyes [21, 22] to identify various steroids eluting from Sephadex LH-20 in organic solvents, we found isatin, [2,3-dihydroindoldione-(2,3)], to be a suitable marker dye for aldosterone in the water-based system.

EXPERIMENTAL

Materials

1. Crystalline d-aldosterone (Aldo), cortisone (E), cortisol (F), corticosterone (B), 11-deoxycortisol (S), deoxycorticosterone (DOC) and Blue Dextran (DB)

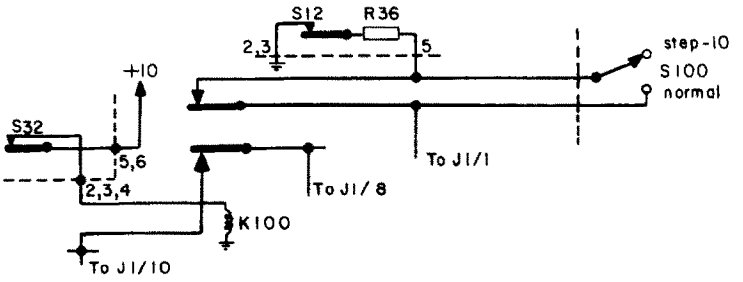

Fig. 1. Circuit diagram of modifications to the LKB Ultrorac fraction collector to permit simultaneous collections from 10 columns. This involved alteration of the internal wiring to selector switches S12 and S32 and the addition of a relay K100 and a switch S100. Symbols refer to the manufacturer's circuit diagram 49-391.

(Sigma Chemical Company, St. Louis, MO) were used without further purification. Solutions of the steroids in absolute ethanol (1 mg/ml) were stored at 4°C.

2. [1, 2-^{3}H]-aldosterone (53 Ci/mmol, Amersham) was purified by chromatography on a Sephadex LH-20 column 30 cm × 1.0 cm i.d. in methylene chloride–methanol (98:2, V/V). The purified tracer was stored in absolute ethanol (0.25 μCi/ml) at 4°C.

3. Sephadex LH-20 (Pharmacia, Uppsala, Sweden).

4. Bovine serum albumin, Cohn Fraction V (Commonwealth Serum Laboratories, Melbourne, Australia).

5. Isatin [2, 3-dihydroindoldione-(2, 3)] (Merck, Darmstadt, G.F.R.).

6. All distilled water used for eluting the columns was degassed prior to the elution.

Instruments

1. Glass columns 60 cm × 1.0 cm i.d. with a sintered glass disc above a Teflon tap at the bottom and a size 14/23 ground glass socket joint at the top were fitted with a size 14/23 socket to cone adaptor incorporating a Teflon tap.

2. The column flow rate was controlled by a Manostat Multichannel Peristaltic Cassette Pump, Cat. No. 75-500-220, with five cassette modules, Cat. No. 72-550-000 (Manostat, New York, N.Y.). Clear tygon autoanalyser pump tubing, 0.065" i.d. was used in the cassette pump (Norwood Scientific Associates, New Jersey).

3. Ultraviolet transmission was measured at 253.7 nm using an LKB Uvicord 4700 and recorded by an LKB 6520 chopper bar recorder (LKB-Produkter AB, Sweden).

4. Column effluents were collected using an LKB Ultrorac 7000 fraction collector (LKB-Produkter AB, Sweden), which was modified to move one rack (ten tubes) at fraction changeover. This was achieved by the circuit alterations shown in Fig. 1. A perspex drop guide 24 cm × 6.0 cm with 7 mm i.d. holes spaced 2.2 cm apart was fitted to the fraction collector for the automated separations.

Packing of columns

Sephadex LH-20 (11.8 g dry weight to give a wet bed height of 60 cm) was allowed to swell overnight in distilled water. A plastic funnel was inserted into the top joint of the column and the column filled with degassed distilled water. The slurry of Sephadex was poured into the funnel and kept in uniform suspension with a mechanical stirrer. When the bed had reached a height of 3 cm, the lower tap was opened and the remainder of the column allowed to pack under gravity. The funnel was removed and a glass fibre disc of Whatman GF/C filter paper 1 cm diameter was positioned on top of the gel in order to protect the bed from disturbance during sample applications. An adaptor incorporating a Teflon tap was attached to the column and the top of the adaptor was fitted with a plastic stopper through which an 18-gauge disposable needle was forced. The needle was connected to the solvent reservoir by plastic tubing (Fig. 2).

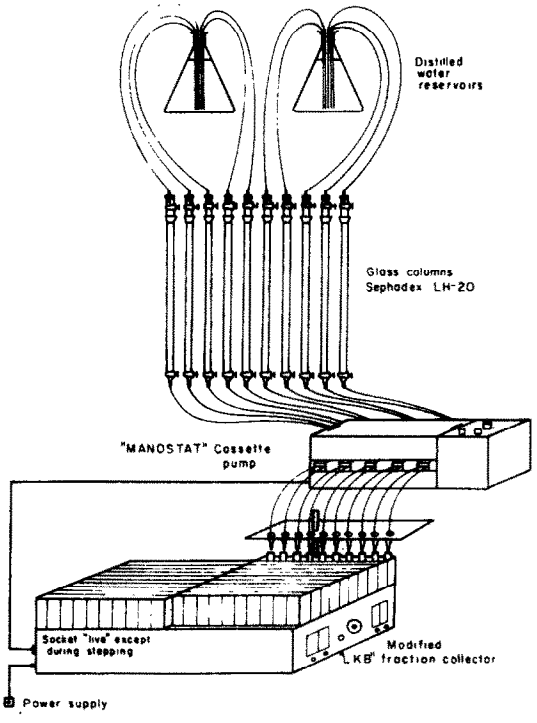

Fig. 2. Arrangement of apparatus for a semi-automated system for running 10 Sephadex LH-20 columns 1.0 cm i.d. × 60 cm in distilled water.

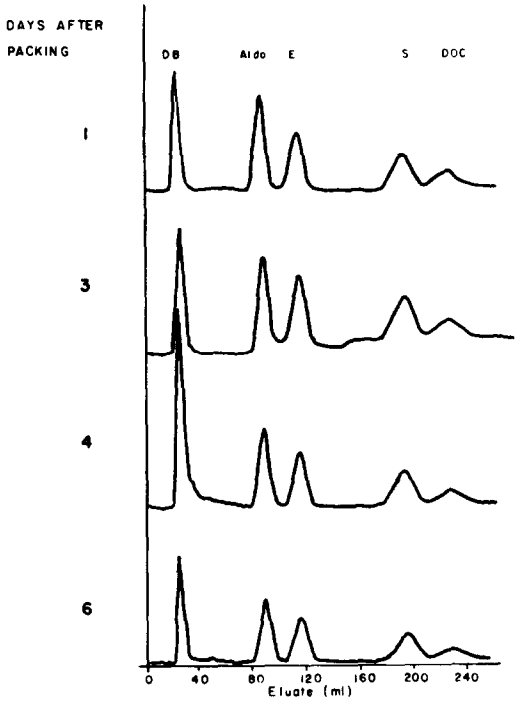

Fig. 3. Elution pattern from a single column of Sephadex LH-20 1.0 cm i.d. × 60 cm eluted with water, 20 ml/h, obtained by measuring U.V. transmission at 253.7 nm 100 μg of each steroid was applied to the column in 0.5 ml of distilled water. (DB = Blue Dextran, Aldo = aldosterone, E = cortisone, S = 11-deoxycortisol, DOC = deoxycorticosterone.)

When the columns were not in use they were washed with a 0.1% solution of sodium azide to prevent bacterial growth. They could be stored this way for long periods (2 years so far in our laboratory) without affecting the gel quality or the steroid elutions.

Automated separations

For running 10 columns the apparatus was set up as shown in Fig. 2 and housed in a constant temperature room at 22°C. The flow rate was controlled at the bottom of the column with a peristaltic pump containing five matched cassettes. Each cassette contained two pieces of autoanalyser pump tubing. The power for the pump was supplied through a socket on the fraction collector which is "live" except during stepping; this arrangement prevents loss of eluate or cross contamination between fractions. The holes in the drilled perspex drop guide were fitted with "Eppendorf" disposable pipette tips to direct the flow from the pump tubing into the tubes. The modification of the fraction collector allowed a maximum of 20 fractions to be collected simultaneously from the 10 columns.

To obtain the steroid elution pattern in Fig. 3, one column was isolated and its eluate diverted to flow through an LKB Uvicord and the ultraviolet transmission was recorded. Before use the column was washed with distilled water (without sodium azide). The sample applied to the column contained 100 μg of each steroid and approximately 500 μg of blue dextran solution in 0.5 ml distilled water. The solvent was removed from the top of the column until the glass fibre disc was exposed. The sample was carefully applied and the pump and fraction collector turned on to allow the sample to run into the gel. The walls of column were washed with small volumes of fresh solvent and these allowed to run in before reconnecting the column to the solvent reservoir. The pump was set to deliver 20 ml/h and 4 ml fractions were collected.

This system allows the columns to run without supervision after the samples have been applied. It was found that the most convenient time to run the columns was overnight. The flow rate for the automated separation was 9.0 ml/h and 55 min. fractions were collected representing a fraction volume of 8.25 ml. From previous calibration of the columns it was possible to predict at what volume the aldosterone peak would elute.

The samples were applied in 0.5 ml of distilled water containing blue dextran, isatin and [^{3}H]-aldosterone recovery marker. The columns were allowed to run overnight and the following morning the two peak fractions were combined and freeze-dried prior to radioimmunoassay.

RESULTS

Separation of steroids

The elution pattern of four reference steroids is shown in Fig. 3. It can be seen from this and Table 1 that aldosterone is eluted from the column first and

Table 1. Elution volumes of steroids from a Sephadex LH-20 column 1.0 cm i.d. × 60 cm in water

Compound	Peak (ml)	Range (ml)
Blue Dextran	25	20–35
Aldosterone	86	76–94
Isatin	100	95–110
Cortisone	112	103–125
Cortisol and corticosterone	127	110–135
11-Deoxycortisol	196	178–210
Deoxycorticosterone	225	215–242

is completely separated from the other steroids. Cortisol and corticosterone are not separated from each other in this system and overlap with cortisone. The elution pattern was highly reproducible over a period of 6 days after packing and this degree of reliability has remained for at least 2 years.

Effect of albumin

The effect of adding albumin to the samples and increasing the sample volume was investigated in the hope of using the system to separate steroids in incubation media without prior solvent extraction. It was found that the elution pattern shown in Fig. 3 was not altered when the sample was applied in 0.5 ml of Krebs–Ringer Bicarbonate containing 4% bovine serum albumin (a total of 20 mg of BSA) or when the steroids were applied in 2.0 ml Krebs–Ringer Bicarbonate containing 4% bovine serum albumin (a total of 80 mg of BSA).

Isatin as a marker dye for aldosterone

Isatin proved to be a convenient marker dye for aldosterone since it elutes immediately after aldosterone without overlapping the peak. Since [^{3}H]-aldosterone co-elutes with unlabelled aldosterone in this system, it was found by radioactive counting that the aldosterone peak was always in the two fractions immediately before the first tube containing the yellow isatin dye.

Recovery of aldosterone

The Sephadex LH-20 columns are used routinely for the determination of plasma aldosterone. For this purpose a preliminary solvent extraction [29] is needed, since the levels of aldosterone in plasma are too low to allow direct sample application. In addition the possible effect of steroid binding proteins in plasma on the chromatographic separation have not been evaluated.

In a typical run of 10 plasmas the recoveries were as follows (mean ± S.D.): solvent extraction step: 83.0 ± 2.1%, column chromatography step: 59.6 ± 6.8% and overall: 49.5 ± 6.3%.

DISCUSSION

Sephadex LH-20 column chromatography using water has important advantages over systems using organic solvents. The problems of gel flotation, solvent purification and the need for solvent resistant equipment are overcome making the system very easy to automate. Using methylene chloride–methanol (98:2, V/V) there tends to be an overlap of the aldosterone peak with cortisone and 11-deoxycortisol [18, 19, 21, 23, 26, 27], but the water based system separates the aldosterone completely from the other steroids investigated. This eliminates any problems of assay interference due to the cross reactivity of these steroids with the aldosterone radioimmunoassay [30].

The order of elution was found to be Aldo, E, F and B, S and DOC. Nowaczynski *et al.*[20] quote a different order for their water system, namely, Aldo, B, F, E, S and DOC. However, in the system reported by Terayama *et al.*[24] the elution order was in agreement with that described in this paper.

This system proved to be convenient and trouble-free. Samples were applied to the columns in the afternoon and left to run unsupervised overnight. The aldo peak was located in the two fractions prior to the first tube containing the yellow isatin dye. The peak fractions could then be freeze dried and used for radioimmunoassay on the same day.

With the modified LKB Ultrorac fraction collector up to ten columns can be run simultaneously. The modifications required were straightforward and resulted in a low cost system which was technically simple and reproducible. We have also found that applying samples to the columns in large volumes and in the presence of albumin does not alter the steroid elution volumes; this permits separation of steroids in incubation media without prior solvent extraction.

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