Journal of Chromatography, 228 (1982) 95—101
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1125

CELITE MULTI-COLUMN CHROMATOGRAPHY FOR THE SIMULTANEOUS SEPARATION OF PROGESTERONE, DEOXYCORTICOSTERONE AND 17α-HYDROXYPROGESTERONE FROM SMALL PLASMA OR TISSUE SAMPLES

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(Received July 10th, 1981)

SUMMARY

A rapid, inexpensive and reliable chromatographic method has been developed for the simultaneous separation of progesterone, deoxycorticosterone and 17α-hydroxyprogesterone on 15-cm Celite columns using *n*-heptane—benzene—methanol—water (100:35:80:20) as mobile phase. Six columns can be run in parallel within an hour with the assistance of nitrogen pressure. There is negligible column-to-column or batch-to-batch variation with consistent high recoveries. Columns are easily and quickly prepared and being inexpensive are disposable which is important when working with radioactivity. Using this method three important corticosteroids can be rapidly and reliably isolated from 20—30 small plasma or tissue samples per day.

INTRODUCTION

With the incomplete specificity of antibodies in the radioimmunoassay of steroids [1], preliminary separations of the individual steroids by chromatography has become an important procedure. This is very much the case with the analysis of progesterone (P), deoxycorticosterone (DOC) and 17α-hydroxyprogesterone (17OHP) where there is close structural similarity and often greatly differing levels in both clinical and experimental situations. Whilst there

0378-4347/82/0000—0000/$02.75

are several studies [2—9] reporting the individual chromatographic separation of P, DOC and 17OHP, more recent systems have concentrated on the simultaneous separation of these unconjugated steroids from plasma [10—12]. The analysis of individual steroid hormones from adrenal tissue extracts is even more complex because of the greater variety of steroid molecules. Preferably however, analyses should be made concurrently on the one sample of plasma or tissue homogenate. A column chromatographic technique using Sephadex LH-20 has been reported for the simultaneous separation of P, DOC and 17OHP [13]. With Celite column chromatography however, only the separation of P from a fraction containing both DOC and 17OHP has been accomplished [14]. Celite column chromatography has several advantages over Sephadex LH-20 chromatography in that the columns are rapid to operate, inexpensive and extremely easy to prepare. A solvent system was selected on the basis of partition coefficients for P, DOC and 17OHP [15] for use with Celite column chromatography. This system provides a rapid, reliable and disposable method for the simultaneous separation of these three precursor steroids, involved in glucocorticoid and mineralocorticoid biosynthetic paths of the adrenal cortex, from the same small samples of plasma or tissue.

EXPERIMENTAL

Materials

Disposable graduated 5-ml glass pipettes (Corning) with an internal diameter of 0.6 cm were used as the supporting microcolumns for the coated Celite. A glass bead (4 mm diameter) was inserted into the tapered end of the pipette as a substitute for a perforated frit used in larger diameter columns. Elution from the columns was prevented by a PTFE adaptor sealing the tapered end of the column. Glassware for extraction, purification, collection and assay of the steroids was disposable negating the possibility of steroid or radioactive contamination. Standard glass scintillation vials (Poulten, Selfe and Lee) were used for collecting eluate from the Celite microcolumns and for liquid scintillation counting with Scintisol (Isolab) as scintillation fluid. Radioactivity was determined by counting in a liquid scintillation spectrometer (Delta 300, Searle) with an efficiency of 50% for tritium. Analytical reagent grade solvents (*n*-heptane, benzene, methanol, *n*-hexane and ethyl acetate) were purchased from E. Merck (Darmstadt, G.F.R.) and used following distillation. Celite 545 (Analytical Filter Aid) was obtained from Johns Manville and was prepared for use by an initial wash with 6 *N* hydrochloric acid for 24 h, followed by a thorough wash with distilled water and then a methanol wash. After drying in a warm oven, the Celite was placed in a muffle furnace at 400° C overnight. The Celite was then stored over absorptive silica in sealed desiccators until required. The tritiated steroids [1,2,6,7-^{3}H]progesterone (100 Ci/m*M*), [1,2-^{3}H]11-deoxycorticosterone (40 Ci/m*M*) and [1,2,6,7-^{3}H]17α-hydroxyprogesterone (100 Ci/m*M*) were obtained from The Radiochemical Centre Amersham, and were prepurified by Celite chromatography.

Extraction of assay buffer, plasma and tissue samples

To each 1 ml of assay buffer [1], plasma and adrenal tissue homogenate (rat

adrenal glands homogenised in physiological saline, 1 ml per 10 mg tissue, using a Potter-Elvehjem homogeniser), 2000 cpm of tritiated P, DOC and 17OHP were added in 0.1 ml of assay buffer as internal standard. After thorough mixing and an equilibration period of 30 min at room temperature, the sample was extracted with 4 ml of ethyl acetate—*n*-hexane (1:1) by vortexing for 30 sec. The lower aqueous phase was then quick frozen in acetone containing chips of dry ice and the solvent layer decanted into a scintillation vial and evaporated under a gentle stream of dry nitrogen in a 45°C water-bath.

Preparation of solvent system and Celite microcolumns

The solvent system for the separation of P, DOC and 17OHP was prepared from 300 ml of *n*-heptane, 105 ml of benzene, 240 ml of methanol and 60 ml of water which were thoroughly mixed by inversion and the two phases allowed to equilibrate for 30 min in a 1 l separating funnel with a PTFE stop-cock. Special care was taken to ensure that all of the water droplets were removed from the heptane—benzene fraction during separation. Following separation, the upper solvent layer (mobile phase) and the lower aqueous layer (stationary phase) were stored in sealed amber-glass bottles. The stationary phase (0.5 ml per g Celite) was thoroughly mixed with purified Celite in a sealed conical flask. The Celite and stationary phase were allowed to equilibrate for 10 min prior to packing. A small glass bead (4 mm diameter) was inserted into the lower end of each column and a glass funnel with a PTFE adaptor was fitted to the top of each column. The Celite (2 g per column) was loaded into the glass funnel and with the use of a glass rod (0.4 mm diameter) a small portion of Celite (ca. 0.2 g) was loosely inserted into each column and then tightly packed. The surface layer of the tightly packed fraction was gently etched before packing each subsequent portion of Celite. The 2 g of Celite was filled to the 0.5-ml graduation on each pipette and gave a microcolumn of Celite 15 cm × 0.6 cm I.D. The remaining volume in the column above the level of the Celite provided for a 3.5-ml solvent reservoir. A 15-cm column of Celite could be packed in 5 min. All elution from the columns was carried out under nitrogen pressure (ca. 0.35 bar) with a flow-rate not greater than 1 ml/min. The columns were all initially eluted with 6 ml of mobile phase leaving 0.5 ml above the level of the Celite and then stoppered with PTFE adaptors until required.

Preferably the columns were used the same day although they could be stored at room temperature for at least five days prior to use.

Technique for chromatographic separation

In order to achieve sharp, reproducible elution profiles for the steroids P, DOC and 17OHP, the equilibrating fraction of mobile phase was eluted to the upper margin of the Celite on the column and then the extracted sample, dissolved in 250 µl of mobile phase, was carefully layered onto the surface of the Celite with a steroid-free disposable glass pipette. The extracted fraction was then eluted to the upper surface of the Celite before addition of more mobile phase. At this stage care must be taken when adding mobile phase to the column to avoid any turbulence at the surface of the Celite. During elution the level of the mobile phase must never fall below the surface of the Celite

as this has a detrimental effect on the steroid elution profile. Appropriate elution fractions were determined by the actual volume of mobile phase pipetted into the column, and were collected into scintillation vials as shown in Fig. 1. For localisation experiments, extracted samples of assay buffer, plasma and tissue homogenate were run on the columns and 0.5-ml fractions were collected into glass test tubes (10 × 50 mm) held in racks of 50 tubes. The eluates were then transferred into scintillation vials containing 10 ml scintillation fluid. Following complete elution of the three steroids, the columns were discarded without re-use.

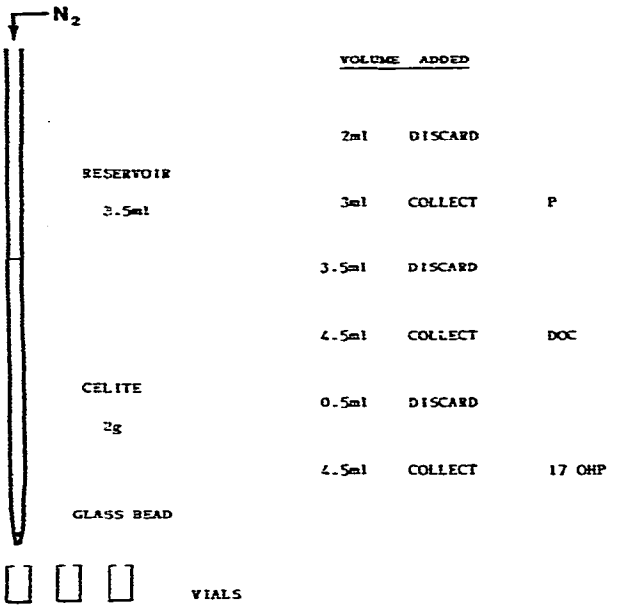

Fig. 1. Diagram of the 15-cm chromatographic column and sequence of volumes of solvent added during a single run with a list of the steroids collected in each of the fractions.

RESULTS

Localisation of the individual steroids

Localisation experiments were carried out with extracts from assay buffer, plasma and adrenal tissue homogenate.

Fig. 2 shows a typical chromatogram of the tritated steroids P, DOC and 17OHP (each containing ca. 2000 cpm) eluted from a 15-cm microcolumn of Celite. The solvent system for the separation of these steroids consisted of *n*-heptane—benzene—methanol—water (100:35:80:20). The chromatographic peaks so obtained were symmetrical and showed good resolution with the troughs between the peaks indicating residual background activity. Table I lists chromatographic elution data including peak volume, fraction collected and volume of the collected fraction. There was no change in the elution pattern for the steroids when extracted from either assay buffer, plasma or adrenal tissue homogenate samples.

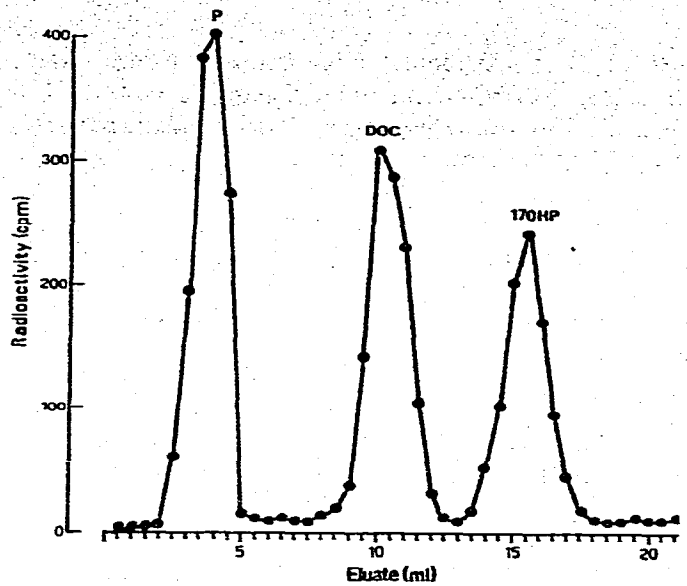

Fig. 2. Chromatogram of tritiated steroids progesterone (P), deoxycorticosterone (DOC) and 17α-hydroxyprogesterone (17OHP) eluted from 15-cm Celite column using *n*-heptane—benzene—methanol—water (100:35:80:20) as solvent system.

Recoveries

During individual localisation experiments the mean recoveries for the tritiated steroids P, DOC and 17OHP, extracted from assay buffer and collected within the fractions indicated in Table I were 92%, 91% and 92% (n = 50), respectively. Routine recoveries for P, DOC and 17OHP, extracted from

TABLE I

CHROMATOGRAPHIC ELUTION DATA OF PLASMA CORTICOSTEROIDS ISOLATED ON 15-cm CELITE COLUMNS

Steroid	Peak volume (ml)	Fraction collected (ml)	Fraction volume (ml)
Progesterone	3.5	2 —5	3
Deoxycorticosterone	11	8.5—13	4.5
17α-Hydroxyprogesterone	16	13.5—18	4.5

adrenal tissue homogenate and collected within the appropriate limits corresponded to 89%, 88% and 87% (n = 50), respectively, which is a slight decrease on assay buffer data. Routine recoveries for plasma of the corresponding steroids were 80%, 78% and 79% (n = 50), respectively. These recovery values for plasma were considerably lower than assay buffer data which is probably due to plasma protein binding. Compared with other chromatographic techniques [4, 6, 16] the recoveries were very high which is important when only small amounts of sample are available.

Reproducibility

The chromatographic profiles of three typical Celite microcolumns, each with a different batch of Celite and solvent phases and carried out over a 6-week period are depicted in Fig. 3. The elution patterns show minimal variation reinforcing the robust nature of the system.

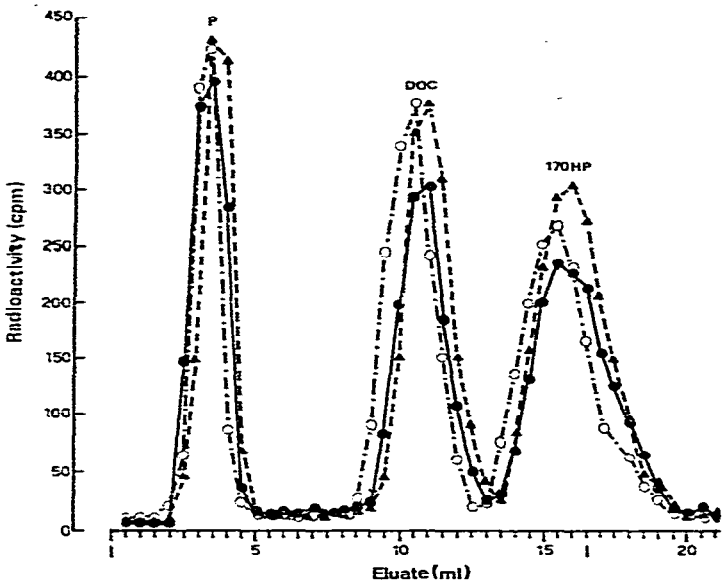

Fig. 3. Reproducibility of elution profiles of the tritiated steroids from three 15-cm Celite columns over a 6-week period.

Practicability

In general, because of the simple preparation required, four to six columns could be packed and equilibrated within 30 min. With six microcolumns interconnected to a nitrogen source, P, DOC and 17OHP could be eluted in parallel from each column within 1 h. Although there was a time difference for the elution of individual columns, this was minimal and had a negligible influence on the elution profiles. The small volumes of eluate collected for each steroid fraction were readily evaporated under dry nitrogen in a water-bath at 45°C. It was possible to process thirty samples by column chromatography over an 8-h period.

DISCUSSION

Despite their very similar polarity and molecular size P, DOC and 17OHP were separated completely and simultaneously from each other with the use of the Celite partition chromatographic system described. The simultaneous separation of these three precursor steroids with Celite chromatography has been achieved with several advantages over Sephadex LH-20 chromatography. These include: (1) ease of preparation; (2) low cost allowing columns to be discarded after single use eliminating the risk of subsequent steroid or radio-

active contamination; (3) rapid nature of the elutionand associated good resolution; and (4) small elution volumes.

In this system testosterone was found to elute in the same fraction as 17OHP. Fortunately there is negligible cross-reactivity of testosterone with 17OHP antisera [12]. Further, the concurrent elution of 17OHP and testosterone is not critical since they usually occur at similar levels. Thus, this leaves an uncontaminated eluate for DOC which usually occurs at much lower levels than 17OHP and testosterone. The elution of more polar steroids by this system proved to be unsatisfactory since they were retained by the Celite stationary phase and extremely high elution volumes were required which resulted in profiles with virtually undiscernible peaks.

The chromatographic system described provides a reliable Celite system for the separation of three important and closely structurally related corticosteroids P, DOC and 17OHP from small plasma or tissue samples prior to radioimmunoassay.

The precise, rapid and inexpensive nature of this method makes it an invaluable technique in clinical diagnosis especially in emergency situations where hormonal profiles are urgently required. Most importantly the very small quantities of sample required will be most applicable to paediatric and experimental endocrinology.

ACKNOWLEDGEMENTS

This study was supported by C.E. Heath and R. Williams Trust Fund. The authors are grateful to Lina Bottazzo for her secretarial assistance.

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