Journal of Chromatography, 276 (1983) 408—412
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1735

Note

Rapid separation of plasma steroids by reversed-phase high-performance liquid chromatography with timed collection of fractions

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(Received February 9th, 1983)

The separation of steroid mixtures using high-performance liquid chromatography (HPLC) has been the subject of numerous studies [1—5]. Often, however, such publications involve gradient analyses, and when the necessary equipment is unavailable the operator wishing to separate steroids of widely differing polarity under isocratic conditions is frequently confronted with lengthy separation procedures, impractical to apply to routine analyses in the clinical chemical laboratory.

We have therefore developed conditions permitting the separation and isolation of the steroids cortisol, androstenedione, testosterone, 17α-hydroxyprogesterone and progesterone from plasma samples in less than 15 min by isocratic reversed-phase HPLC. The incorporation of a partial purification of plasma extracts prior to HPLC eliminates the need of frequent regeneration of the column by removing late-eluting UV-absorbing components.

Measurement of these steroids is most useful for the diagnosis of congenital hyperplasia due to 21-hydroxylase deficiency and offers an index for the adequacy of treatment of such patients.

EXPERIMENTAL

Materials and methods

[^{3}H]Cortisol (56.0 Ci/mmole), [^{3}H]androstenedione (90.0 Ci/mmole), [^{3}H]testosterone (50.4 Ci/mmole), 17α-[^{3}H]hydroxyprogesterone (50.0

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0378-4347/83/$03.00

Ci/mmole) and [^{3}H]progesterone (101.0 Ci/mmole) were obtained from The Radiochemical Centre, Amersham, Great Britain. Portions of each steroid were diluted in 0.2% (v/v) aqueous ethylene glycol so that a 100-μl aliquot contained approximately 5000 cpm. Non-radioactive steroids were obtained from Steraloids, Wilton, NH, U.S.A. Radioactivity was measured in a Packard Tri-carb liquid scintillation counter (Packard Instrument, Downers Grove, IL, U.S.A.) using Aqua Luma^R scintillation liquid (Lumac Systems, Titusville, FL, U.S.A.). Counting efficiency was 48—50%.

Liquid chromatography was performed using a Model 6000A solvent delivery pump, a U6K injector, a Model 480 LC spectrophotometer and a Model 730 data module, all from Waters Assoc., Milford, MA, U.S.A. A Hypersil 5 ODS column (150 × 4.6 mm), purchased from Chrompack, Middelburg, The Netherlands, was used for the analytical separations. A guard column, packed with pellicular C_{18} material was used to protect the analytical column. The solvent composition was methanol—tetrahydrofuran—water (3:2:5, v/v) at a flow-rate of 1 ml/min, resulting in a column pressure of 14 MPa.

Retention times were determined using authentic standards (10 ng each) and were reproducible to within 0.2% over a 4-h period.

Plasma samples were taken from pooled plasma, obtained from healthy adults (male and female blood donors).

Sep-Pak^R C_{18} cartridges were obtained from Waters Assoc. and were attached to Eppendorf combitips 12.5 ml (Eppendorf, Hamburg, G.F.R.) to facilitate sample application and elution.

Extraction procedure

Following incubation with tritiated steroids (approx. 5000 cpm in 100 μl of 0.2% aqueous ethylene glycol) for 30 min at 37°C, 2-ml plasma samples were extracted with diethyl ether (16 ml). After evaporation of the extracts the residues were taken up in 5 ml of 0.9% (w/v) aqueous sodium chloride and were kept at 37°C for 15 min to dissolve the steroids. Upon cooling to room temperature the suspensions were applied to Sep-Pak C_{18} cartridges which had been washed previously with methanol (10 ml), water (10 ml), and 0.9% aqueous sodium chloride (10 ml). The cartridges were washed with sodium chloride solution (5 ml) and water (10 ml) and then eluted with methanol—tetrahydrofuran—water (3:2:5, v/v; 7.5 ml). The eluates from the cartridges were concentrated to about 4 ml under a stream of purified air at 37°C and were then extracted with diethyl ether (2 × 10 ml). The combined ether extracts were evaporated and dissolved in 50 μl of methanol—tetrahydrofuran—water (3:2:5, v/v) for subsequent liquid chromatography.

Fraction collection

The Model 730 data module was used to actuate a three-port solenoid switching valve 24 VDC (Pharmacia, Uppsala, Sweden), thus allowing the timed collection of fractions of the eluate from the LC column into an LKB 2070 Ultrorack fraction collector. The circuit diagram of the interface (home-made) required to operate the valve and fraction collector is shown in Fig. 1. A brief explanation of its principle of operation follows.

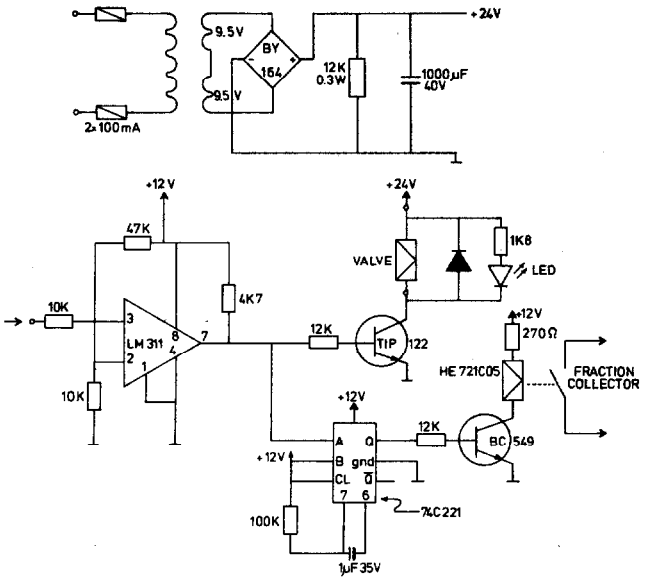

Fig. 1. Circuit diagram of interface and connections between data module, fraction collector (pins 10 and 13 of external input) and valve. K = kΩ, 4K7 = 4700 Ω, 1K8 = 1800 Ω.

The 12 V d.c. output on the data module is used to feed the interface. The timed event output (normally + 5 V d.c.) drops to ground potential upon activation, thus triggering the voltage comparator LM 331 which in turn triggers transistor TIP 122 and the valve is actuated. When the timed event output reverts to + 5 V d.c. the valve returns to its normal position, diverting the eluate from the LC column to a waste container. Through IC SN74C221 triggering transistor BC 549 the voltage comparator activates the Reed relay which causes the fraction collector to step. A light-emitting diode is connected in parallel to the valve's coil and provides visual inspection of the switching of the valve.

RESULTS AND DISCUSSION

Binary mixtures of methanol, acetonitrile or tetrahydrofuran and water were unsuccessful in resolving the steroids of interest under isocratic conditions within 15 min analysis time. Therefore ternary mixtures were used to determine optimal LC conditions since such mixtures have been shown [6—8] to possess powerful selective properties. Although a radially compressed C_{18} column (100 × 8 mm, 5 µm particle size; Waters Assoc.) in combination with acetonitrile—tetrahydrofuran—water (4:1:5, v/v) was satisfactory with respect to resolution and analysis time, the Hypersil 5 ODS column in combination with methanol—tetrahydrofuran—water (3:2:5, v/v) was selected because, due to a smaller internal diameter and lower flow-rate, peak volumes were smaller (1 ml vs. 3—4 ml). This will undoubtedly be advantageous in off-line quantitation, e.g. radioimmunoassay.

The extent to which cross-contamination of fractionated steroids might occur was determined in several trial experiments. Following measurement

of retention times of authentic standards (non-radioactive, 10 ng of each) and correcting for the delay time from detector to collector, the data module was programmed to collect fractions from 10—30 sec before, until 40—60 sec after, the corrected retention times thus obtained. Mixtures of standards, charged with separate ^{3}H-labelled steroids (approx. 5000 cpm) were then chromatographed and the percentage distribution of radioactivity was determined across collected fractions from each analysis. If necessary, the time interval for collecting individual fractions was then adjusted until cross-contamination was less than 5% (Fig. 2).

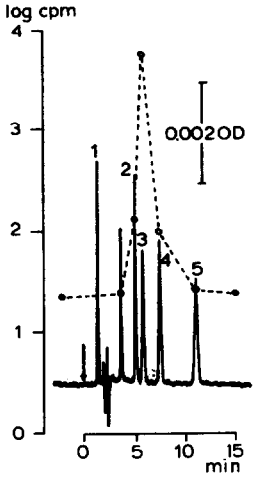

Fig. 2. HPLC separation of a mixture of steroids charged with [^{3}H]testosterone on a Hypersil 5 ODS column (150 × 4.6 mm) using methanol—tetrahydrofuran—water (3:2:5, v/v) as the mobile phase at 1 ml/min. Absorbance measured at 240 nm. Injected volume 45 μl (10 ng of each component). (o- - - -o), amount of radioactivity contained in each fraction collected (note logarithmic scale). Peaks: 1 = cortisol, 2 = androstenedione, 3 = testosterone, 4 = 17α-hydroxyprogesterone, 5 = progesterone.

Extraction of plasma steroids was originally conducted as described by Cannell et al. [9]. In short, this procedure involves loading Sep-Pak C_{18} cartridges with plasma samples diluted (1:10) with acetate buffer and eluting the adsorbed steroids with methanol. Although this method gave satisfactory results on 1-ml plasma samples we found the HPLC column to be rapidly contaminated, necessitating frequent regeneration. We therefore modified the method of Cannell et al. by loading Sep-Pak cartridges with ether extracts instead of whole plasma and eluting the adsorbed steroids with methanol—tetrahydrofuran—water (3:2:5) in place of methanol. In this way injection of methanol (1.5 ml), following elution of the progesterone peak, adequately cleaned the column between separations. Since recoveries of steroids were lower, due to the incorporation of two solvent extraction steps, 2-ml plasma samples were used (Fig. 3).

Recoveries of the individual steroids through the extraction procedure ranged from 65% (cortisol) to 95% (testosterone), as determined from radioactivity measurements. With the exception of androstenedione, recoveries

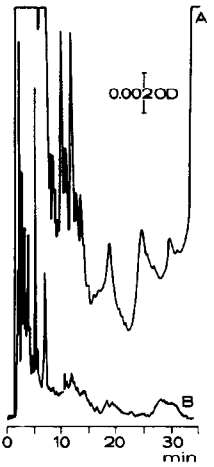

Fig. 3. Liquid chromatogram of Sep-Pak eluates of plasma samples. (A) 1 ml of whole plasma, eluted with methanol. (B) Ether extract of 2 ml of plasma eluted with methanol—tetrahydrofuran—water (3:2:5, v/v). Conditions as in Fig. 2.

in the HPLC fractionation procedure were between 75% and 80%. The low recovery of androstenedione (56%) was the price paid for keeping cross-contamination <5%. Therefore the overall recovery of androstenedione averaged 45% while the other steroids showed recoveries of 55—65%.

CONCLUSIONS

The HPLC fractionation of plasma extracts purified on Sep-Pak C_{18} cartridges as described in this paper provides a rapid and simple means of isolating the steroids cortisol, androstenedione, testosterone, 17α-hydroxyprogesterone and progesterone. Yields are more than adequate for subsequent quantitation by radioimmunoassay. The extraction and purification procedures can easily be applied to large sets of plasma samples and the HPLC fractionation procedure allows rapid, consecutive separations suitable for automation.

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