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Note

Determination of small amounts of corticosterone in high-level lipid plasma by means of thin-layer chromatography

R. SABLE-AMPLIS, R. AGID and D. ABADIE

E.R.A. C.N.R.S. No. 412, Institut de Physiologie, Rue François Magendie, 31078 Toulouse Cedex (France)

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Plasma corticosterone levels are relatively low in common laboratory animals. Fluorimetric techniques are simple and reliable when the plasma contains a normal amount of lipid. The corticosterone concentration in rabbit plasma is in the range of 1–5 μg per 100 ml. The normal mean value for total lipid is about 300 mg per 100 ml and this level may be ten times higher in animals that have been adrenalectomized or bled daily, and even higher in cholesterol-fed animals. Several methods have been described for avoiding the influence of interfering lipids when corticosterone is determined. Purification by two-dimensional chromatography is excellent but allows not more than one determination per plate; therefore, a simultaneous standard cannot be run. We describe in this paper a simple and effective method that has the advantages of two-dimensional chromatography but also permits the determination of several samples on the same plate.

EXPERIMENTAL

Apparatus

A Desaga (Heidelberg, G.F.R.) applicator was used to coat the 20 × 20 cm glass plates with silica gel H (Merck, Darmstadt, G.F.R.). The samples were applied with a Manostat microburette on the activated plates (0.5 h at 100°). The plates were developed horizontally in a Desaga "Sandwich" chamber. Corticosterone was made visible on the plates with iodine vapour in a special Plexiglass box made in this laboratory (Fig. 1).

Spectrofluorimetric readings were made with a Safas spectrofluorimeter. All glassware was washed with detergent, rinsed with ethanol and dried before use.

Reagents

Analytical grade *n*-hexane, cyclohexane, ethyl acetate, toluene, chloroform, sulphuric acid and 0.1 *N* sodium hydroxide solution were used. Methanol, ethanol and methylene chloride were freshly redistilled before use. The fluorescence reagent was that of Zenker and Bernstein[1]. Corticosterone standard was purchased from N.B.C. (Cleveland, Ohio, U.S.A.). The standard solution was prepared in ethanol (10 μg per 100 ml). This stock solution was diluted with water just before use so as to contain

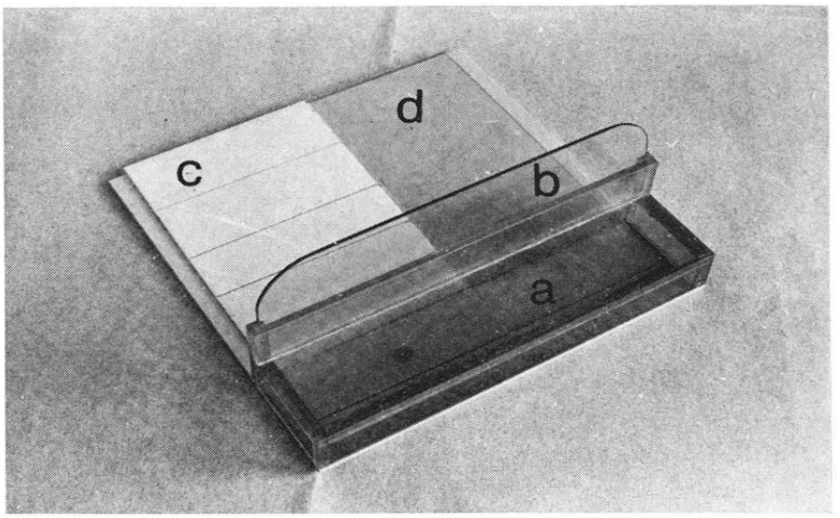

Fig. 1. Apparatus for the localization of the concentrated corticosterone standard. a, Portion of the plate containing concentrated standard placed in the iodine vapour; b, Plexiglass partition; c, portion of the plate containing experimental samples, blank and diluted standard; d, portion of the plate in which the first two developments were carried out. The silica gel has been removed.

0.05 or 0.1 μg per 100 ml, and 1 ml was treated as the plasma. The silica gel H was washed with warm methanol and dried before use.

Extraction

A 1-ml volume of plasma and 10 ml of methylene chloride were shaken with a Vortex mixer. Following centrifugation, the organic phase was mixed (after separation) with 0.2 ml of 0.1 *N* sodium hydroxide solution, then washed with 1 ml of distilled water. The methylene chloride extract was separated and evaporated to dryness under a stream of nitrogen. Diluted standard solutions were extracted by the same procedure. The dry residues from plasma, standard and blank extracts were dissolved in 50 μl of methylene chloride.

Chromatographic procedure

The plates were activated at 110 for half an hour. The samples were spotted on a line in the middle of the plate and not at the bottom. At one end of the middle starting line, a 10–100-fold concentrated solution of corticosterone was spotted. This spot allowed us to detect only the concentrated standard and to avoid contact of the whole plate with the iodine vapour (Fig. 1). To facilitate scraping, the silica gel was previously divided into 2-cm lanes before spotting.

Three successive developments were then carried out using the following systems:

System I: *n*-hexane–ethyl acetate (4:1).

System II: ethyl acetate–cyclohexane–toluene (10:10:1).

System III: ethyl acetate–chloroform–water (90:10:1).

After each development, the plate was dried carefully at room temperature.

The first two developments ran in the same direction, their aim being to eliminate most of the non-corticoid substances (Fig. 2). Then the silica gel was removed from the front approximately 1 cm from the spotted starting line. The cleared area was washed with ethanol and covered with filter paper previously washed with solvent system III. After a 180° rotation of the plate, the third development was carried out in a direction opposite to the first two, the filter paper starting the solvent flow.

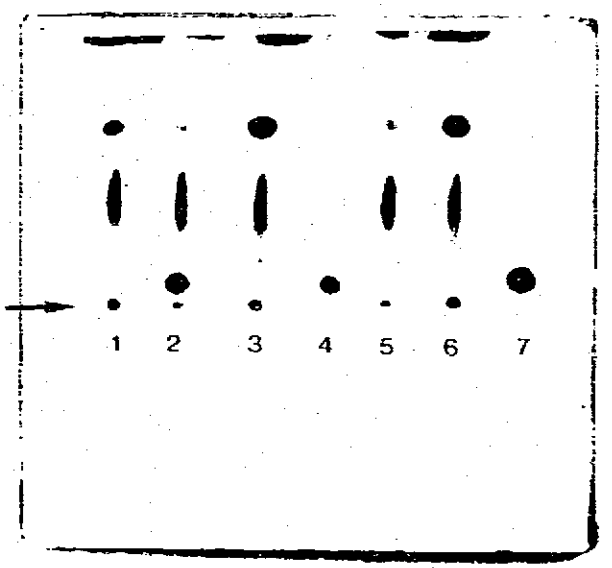

Fig. 2. Two-dimensional thin-layer chromatogram. The arrow indicates the initial starting line. The samples were spotted on a middle line. 1 = Mixture of lipid standards (lecithin, cholesterol, triglycerides, cholesterol esters); 2 = plasma extract of normal rabbit plus corticosterone (5 μg); 3 and 6 = plasma extract of a rabbit on high cholesterol diet; 4 = corticosterone standard (5 μg); 5 = plasma extract of normal rabbit; 7 = concentrated corticosterone solution used for localization by iodine vapour. After the first two developments most of the non-corticoid substances were eliminated.

At the end of the last development, the corticosterone ran 3 cm from its start. We localized the steroid by means of iodine vapour, which was allowed to contact the plate only in the region occupied by the concentrated corticosterone standard. After localization, the corticosterone areas were scraped off and extracted with methanol.

RESULTS AND DISCUSSION

The method gave good accuracy and reproducibility when the concentration in plasma was above 0.02 μg/ml. A standard solution was prepared to contain 0.05 μg/ml; one aliquot was measured by the direct fluorescence method and another by the chromatographic technique described here. The first method (five determinations) gave results averaging 77 ± 3 (standard error) fluorescence units, while five determinations using the chromatographic method gave an average of 86 ± 7 fluorescence

units. There was no statistical difference (Student *t*-test) between the averages for the two techniques.

The chromatographic technique was used with rabbits on a high cholesterol diet. After a few days on this diet, the spectrofluorimetric assay gave irregular and unusually high values for corticosterone (higher than 20 μg per 100 ml of plasma); with our technique we found the values shown in Table I.

TABLE I

PLASMA CORTICOSTERONE LEVELS (μg PER 100 ml) IN RABBITS ON A HIGH CHOLESTEROL DIET

Rabbit No.	*Before diet*	*Diet for 1 week*	*Diet for 15 days to 2 months*
1	8.3	4.5	0.8
2	2.5	3.3	Trace
3	3.0	10.0	1.2
4	3.3	5.0	2.0

To conclude, the method described permits the separation and determination of less than 0.05 μg of corticosterone. It gives results as precise as those of two-dimensional chromatography. In addition, it permits three samples plus a standard to be assayed in duplicate on a single plate.

REFERENCE

1 N. Zenker and E. Bernstein, *J. Biol. Chem.*, 231 (1958) 695.