Microdetermination of Acetylable Steroids in Plasma.* (22876)

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Many of the steroid analytical methods described in the literature have contributed to the present knowledge of steroid metabolism; however, they have been limited in application due to relatively low sensitivity and specificity. Methods based upon chromogenic procedures(1-3) are generally specific for molecular groupings on the steroid molecule, not individual steroids. Polarographic(4) and fluorometric(5) procedures are relatively specific, but are applicable to only a limited number of steroids. Paper chromatography procedures(6,7), although relatively specific for a wide variety of steroids, have presented problems in quantitation.

The method described here employs a combination paper chromatography-radioactive isotope technic which gives high sensitivity and specificity in an analytical range not heretofore possible. In this method, steroids with 3, 20, or 21-hydroxyl groups (includes all corticosteroids) are acetylated with radioactive acetic anhydride-1-C^{14} (S.A. 2 millicuries millimol)† in pyridine. After chromatography, the radioactivity of the steroid acetates is determined directly from the paper chromatogram. C^{14} acetylation for identification of steroids has previously been employed(8), but neither details of the procedure nor the quantity of steroid acetylated has been reported. Five to 10 ml of plasma are extracted with chloroform, the chloroform evaporated *in vacuo*, and the fatty residue partitioned between hexane and 75% methanol. The hexane is discarded and the methanol evaporated and re-extracted with chloroform. The extract is dried and acetylated with 700 μg of radioactive acetic anhydride in pyridine overnight at room temperature. This fraction contains the "free" steroids. A beta-glucuronidase hydrolysis(9) of the plasma residue is carried out and the hydrolysate extracted with chloroform, evaporated and acetylated in the above manner. This fraction contains the steroid glucuronides. After acetylation is completed, the excess acetic anhydride is removed by a small stream of nitrogen. Two sodium hydroxide traps are employed in a train to catch the excess radioactive acetic anhydride. The dried residue is dissolved in a small quantity of chloroform-methanol 1:1 and chromatographed on paper according to the method of Zaffaroni(6). After chromatography, the paper is dried in air and passed through an automatic strip counter. The radioactive area on the recording paper is measured by a planimeter to determine the quantity of steroid. To determine the quantity of acetic anhydride required to react quantitatively with a known quantity of steroid added to the plasma, radioactive 4-C^{14} labeled corticosterone and tetrahydrocortisone in quantities of 1 and 1.4 μg respectively were added to 5 ml sample of plasma and carried through the above procedure. The samples were acetylated with various quantities of non-radioactive acetic anhydride. Fig. 1 illustrates data which show quantitative recovery when quantities of acetic anhydride greater than 500 μg were employed. The chromatographic system employed varies with the steroid to be determined. The isolation and quantitation of a single hormone—cortisol—will be taken as an example. After the plasma has been extracted with chloroform‡, and the extract dried and acetylated, 100 μg

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† This can be diluted in accordance with the quantity of steroid acetlyated.

‡ An alternate procedure for extraction of cortisol from plasma has proven advantageous. The plasma is first extracted with heptane to remove fat and other low polar substances. A subsequent extraction with chloroform yields a quantitative recovery of cortisol essentially free from other interfering compounds. Steroids more polar than cortisol (Tetrahydrocortisol) may be extracted quantitatively by substituting benzene for heptane and subsequently extracting with chloroform.

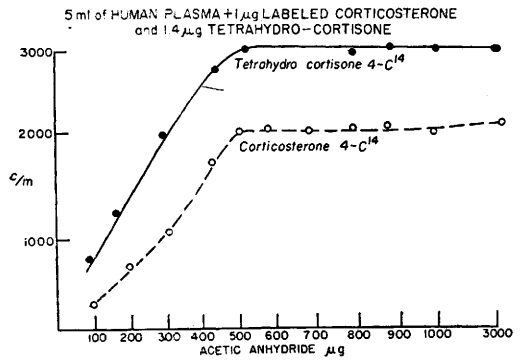

FIG. 1. Quantitative recovery of radioactive corticosterone and tetrahydro-cortisone with various amounts of non-radioactive acetic anhydride.

of non-radioactive carrier cortisol acetate are added. The mixture is then chromatographed in the benzene-formamide system for 5 to 8 hours. After the paper is dried, the paper chromatogram is analyzed for radioactivity by means of a recording strip-flow counter; the position of the added cortisol is detected with a Haines-type scanner(10). The cortisol area is then eluted with methanol and the specific activity of the eluent determined as follows: An aliquot of the eluent is analyzed spectrophotometrically to determine the total quantity of alpha-beta unsaturated ketone present. The quantity of steroid in the plasma as represented by the radioactivity is then determined by the following equation:

$$\frac{F \times A \times M}{B \times C} = \mu g \text{ steroid in plasma sample.}$$

F = the beta ray self absorption factor of the paper. This factor is very constant for any particular system and paper, but must be determined for each system.§ In our present system, this is 6.12.

A = radioactivity area in c/m

M = molecular weight of steroid in μg.

B = fraction of added steroid recovered. In our system this has been 0.7-0.9

C = specific activity of acetyl radical in c/m/μmole. This must be determined for each counting system.

Results. When known quantities of cortisol are acetylated with radioactive acetic anhydride, a linear curve between corrected counts per minute and μg cortisol is obtained; this allows direct reading from a graph.

A simple procedure is employed to confirm the identity of an unknown compound. After the specific activity is determined as above, the mixture is oxidized with CrO_3(6), and rechromatographed in the same system. If the substances are identical they migrate at the same rate on the paper and the specific activity is constant(11). This technic is illustrated in Fig. 2. The specific activities were found to be identical in both the untreated and oxidized steroid.

Six samples of plasma were obtained at 3:00 p. m. from normal subjects and analyzed for cortisol content. The mean value determined corresponds to 0.86 microgram per 10 ml of plasma with a standard deviation of 0.042. 410 ml of pooled normal plasma were carried through the same procedure, using an excess of radioactive acetic anhydride. From this plasma 36.2 μg of cortisol acetate were isolated and identified by both radioactive (11) and chemical(6) methods. This level corresponds to 0.88 μg/10 ml of plasma, which is comparable with those obtained by the micro-method technic.

Other compounds have been isolated and identified by the same procedure: cortisone from the free fraction, and tetrahydrocortisol, tetrahydrocortisone and pregnandiol from the glucuronide fraction. Additional identifications are in progress. Quantities of less than 0.1 μg steroid have been detected by this method. The sensitivity depends on the specific activity of the labeled acetic anhydride used. Sensitivity is greater for steroids having more than one acetylable hydroxyl group. The application of this method to studies of fluids and tissues in diseased states will be reported elsewhere.

Summary. A method is described which employs a combination of paper chromatographic and radioactive isotope technics. It is applicable to a wide number of hydroxylated steroids and is specific and sensitive to less than 0.1 μg quantities of steroids. The sensitivity is dependent upon the number of hydroxyl groups and specific activity of the radioactive acetic anhydride which is used to

§ To be published.

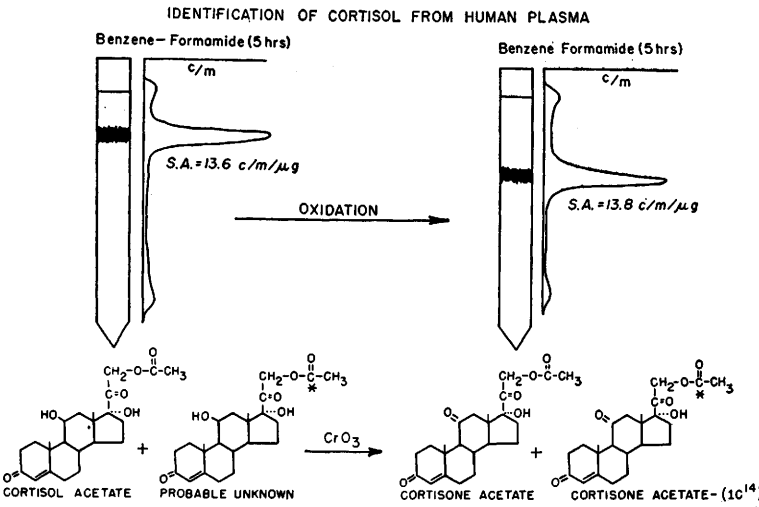

FIG. 2. Chromatographic procedure for identification of cortisol. Specific activity (S.A.) of cortisol acetate after oxidation remains constant.

label the steroid. It has been applied successfully to the analysis of steroids in blood, urine, and a variety of tissues.

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