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Separation of rat corticosteroids on Sephadex LH-20

In the past, the purification steps required for an accurate analysis of isolated adrenal corticosteroids common to the rat have been too numerous and time-consuming to allow an individual to analyze a large number of samples. In addition, known 18-hydroxylated corticosteroids have been shown to be unstable[1,2], and it is therefore desirable and perhaps necessary to have available separation and purification methods which avoid drying and eluting steps usually required with paper and thin-layer systems. With the availability of commercial lipophilic Sephadex LH-20 (Pharmacia, Piscataway, N.J.) the rapid separation of some steroids has been reported[3,4], but to our knowledge this approach has not been exploited with the idea of maximizing overall yields, at submicrogram levels, of corticosteroids biosynthesized and secreted by the rat adrenal gland.

Sephadex LH-20 (500 mg) was allowed to swell in excess 80% methanol overnight. A 5-ml disposable pipet (Corning No. 7077) containing one 3-mm glass bead at the tip served as a column. The Sephadex slurry was added to the column and allowed to settle by gravity. Between 40 to 50 ml of the eluant, benzene–cyclohexane (2:1), equilibrated overnight with 80% methanol, were passed through each column until the excess methanol used to swell the Sephadex was washed from the column. At this point, columns so prepared could be stored by submerging them in benzene–cyclohexane until ready for use. The chromatographic separations were done at room temperature and atmospheric pressure.

Tritiated 21-hydroxypregn-4-ene-3,20-dione (DOC), 11β,21-dihydroxypregn-4-ene-3,20-dione (corticosterone), 18,21-dihydroxypregn-4-ene-3,20-dione (18OH-DOC) and 11β,21-dihydroxy-18-oxopregn-4-ene-3,20-dione (aldosterone) were each applied separately, in 0.2 ml of eluant, to different Sephadex columns. Fractions of 1 ml were collected and the radioactivity was measured by liquid scintillation counting. The elution patterns represented in histogram form (Fig. 1) indicate that between 95–100% of the applied corticoids were recovered.

In order to determine the purity of the peaks and the capacity of the columns when tissue extracts were used, the four tritiated corticoids mentioned above were mixed with 10 μg of their respective inert carriers and added to a 1-ml rat adrenal homogenate (15 mg/ml) which contained less than 200 ng of total endogenous corticosteroids. The tissue sample was extracted[5] and purified on the Sephadex LH-20 column. The purity of the corticosteroids eluted from the column was determined in three different chromatographic systems. Small aliquots from each steroid peak (see brackets in Fig. 1) were analyzed on a BUSH-B_6 paper chromatography system[6] and a thin-layer system: toluene–propylene glycol on kieselguhr (Brinkmann Instruments, Inc.) impregnated by predevelopment in 6% propylene glycol in acetone. Additional aliquots from the DOC, corticosterone, 18OH-DOC and aldosterone peaks were oxidized with periodic acid and the etiolactones and etioacids formed were separated on modified BUSH-type systems[5]. After scanning all strips for tritium in a Vanguard Autoscanner each chromatogram was cut into small sections, eluted in ethanol and

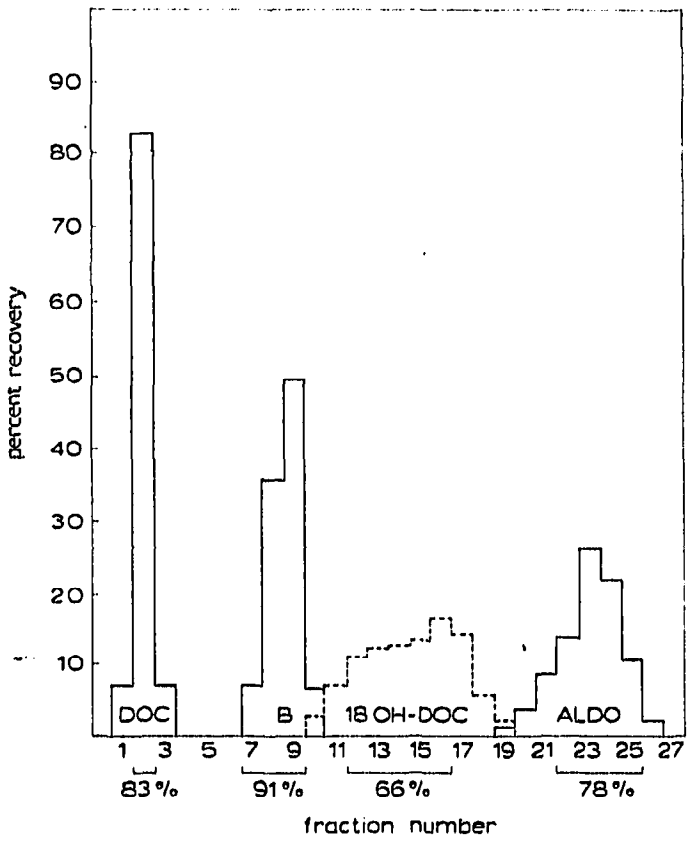

Fig. 1. Elution curves of 21-hydroxypregn-4-ene-3,20-dione (DOC), 11β,21-dihydroxypregn-4-ene-3,20-dione (B), 18,21-dihydroxypregn-4-ene-3,20-dione (18OH-DOC) and 11β,21-dihydroxy-18-oxopregn-4-ene-3,20-dione (ALDO) on Sephadex LH-20 using 80% methanol as the stationary phase and benzene–cyclohexane (2:1) as the mobile phase. 1-ml fractions were collected. Bracketed areas represent portion of peak analyzed for purity.

counted in a liquid scintillator. The results clearly indicated that DOC, corticosterone, 18OH-DOC and aldosterone peaks were pure, and contained no tritium cross-contaminates.

The percent recovery of the inert carrier was assayed in fractions 2 (DOC) and 22–23 (aldosterone) colorimetrically by the blue tetrazolium reaction[7] and fraction 7–9 (corticosterone) by fluorometry[8] and the 18OH-DOC fraction, 12–15, was analyzed by the Porter–Silber reaction[9]. While some loss of steroid was to be expected during the extraction procedure, the actual amount recovered in each peak was only 5–10% less than that found when radioactive tracers were applied directly to the columns.

Application of the above method to actual experimental work in our laboratory[10] has demonstrated that a large number of samples containing physiological levels of corticosteroids present in adrenal tissue and plasma can be rapidly extracted and then separated and purified on Sephadex LH-20 in a single afternoon. Furthermore, the low cost of preparing the columns makes it possible to dispose of each column after a single use and thus eliminate the chances of contaminating other samples.

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