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Note

Micro-assay for sheep 11β-hydroxylase activity using high-performance liquid chromatography for steroid analysis

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During an investigation of plant products from the shrub *Salsola tuberculata* that affect adrenal steroid hormone biosynthesis[1], an assay for the conversion of deoxycortiosterone to corticosterone by the adrenal 11β-hydroxylase enzyme system (cytochrome P-$450_{11\beta}$) was required for screening purposes. Since a large number of samples containing small amounts of highly labile compounds had to be screened, none of the assay procedures previously reported for this conversion[2–7] was suitable.

The main problems encountered with previously published methods were: time-consuming purification of relatively large amounts of enzymes required for multiple conversion assays, the separation of corticosterone, deoxycorticosterone, cofactors and inhibitors after incubation and accurate quantitation of steroids. The latter two problems were mainly caused by the interference of added plant extract.

Several studies by other researchers[2–5] showed that the enzymatic conversion of deoxycorticosterone to corticosterone by the adrenal mitochondrial 11β-hydroxylase system requires the presence of oxygen, adrenodoxin reductase (ADXR), adrenodoxin (ADX), cytochrome P-$450_{11\beta}$, NADPH ands an NADPH regenerating system such as glucose-6-phosphate, glucose-6-phosphate dehydrogenase and magnesium chloride. The new method is based on the incubation of deoxycorticosterone with this reconstituted system in a total volume of 200 μl.

After incubation steroids were separated and quantitated in one step using isocratic high-performance liquid chromatography (HPLC). Separation was achieved on a normal-phase silica column and detection of the steroids was done with a fixed-wavelength UV detector at 254 nm. Recording and integration of chromatograms were performed with the aid of the data processing facility of the chromatograph.

The use of HPLC allowed the rapid high-resolution separation of steroids in the incubation medium. Interference by test compounds with separation and quantitation, encountered with thin-layer chromatographic (TLC) separation and radiometric as well as fluorometric detection, was thus eliminated.

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EXPERIMENTAL

Materials

Glucose, glucose-6-phosphate dehydroghenase and NADPH were purchased from Sigma (St. Louis, MO, U.S.A.). Deoxycorticosterone (4-pregnen-21-ol-3,20-dione) and corticosterone (4-pregnen-11β, 17-diol-3,20-dione) were purchased from Steraloids (Wilton, U.S.A.). HPLC-Grade methanol was purchased from E. Merck (Darmstadt, F.R.G.). Analytical-grade methylene chloride from Waters (Milford, MA, U.S.A.) had to be freshly glass distilled just before use. All other chemicals were of reagent grade and were used without further purification.

The conversion assay

The cytochrome P-450 preparation was prepared from sheep adrenals by the method of Cheng and Harding[4]. A dilute ADX–ADXR-containing preparation was obtained by the method of Omura and Sato[2] as modified by Bjorkhem and Karlmar[3] and Omura *et al.*[8]. The dilute preparation was concentrated by a method previously published[9].

The 11β-hydroxylase assay was carried out in eppendorf tubes (1.5 ml). The lid of each tube contained two hypodermic needles to supply oxygen. A standard assay mixture (200 μl) consisted of the following: cytochrome P-450 (2.23 μM), ADXR–ADX preparation (1320 units/ml) deoxycorticosterone (20 nmol in 5 μl propylene glycol), glucose-6-phosphate (48 μM), glucose-6-phosphate dehydrogenase (1.875 units/ml) and magnesium chloride (6 mM) in phosphate buffer (0.05 M, pH 7.4). Solutions of test compounds were added, and the volume compensated for by the omission of the appropriate amount of buffer to maintain a total volume of 200 μl for the assay mixture. The mixture was pre-incubated at 37°C for 8 min under oxygen. The reaction was initiated by the addition of NADPH (3 mM in phosphate buffer), continued under oxygen for 5 min and terminated by the addition of methanol (200 μl).

Sample preparation

Steroids were extracted with dichloromethane (3 × 200 μl) and the extracts dried under nitrogen. The residue was taken up in dichloromethane, filtered through an 0.2-μm membrtane and subjected to HPLC analysis. More than 90% of the steroids were extracted by this method.

HPLC of deoxycorticosterone and corticosterone

Chromatography was carried out with a Waters liquid chromatograph consisting of a Model M6000 solvent delivery system, a UK6 injector, a Model 440 fixed-wavelength detector and a Model 840 data station. Steroids were separated on a waters Radial-Pak 10-μm silica column (150 × 10 mm) equipped with a guard column at 25°C with a dichloromethane–methanol (97:3) mixture as mobile phase at a flow-rate of 1.5 ml/min. The column effluent was monitored at 254 nm.

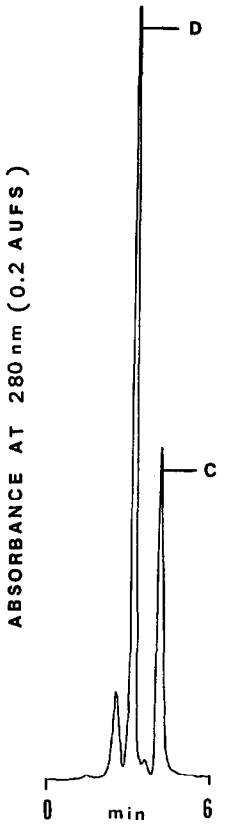

Fig. 1. HPLC separation of deoxycorticosterone (D, 0.5 nmol) and corticosterone (C, 0.25 nmol) on a 10-μm silica column at 25°C. Mobile phase: dichloromethane–methanol (97:3). Flow-rate: 1.5 ml/min.

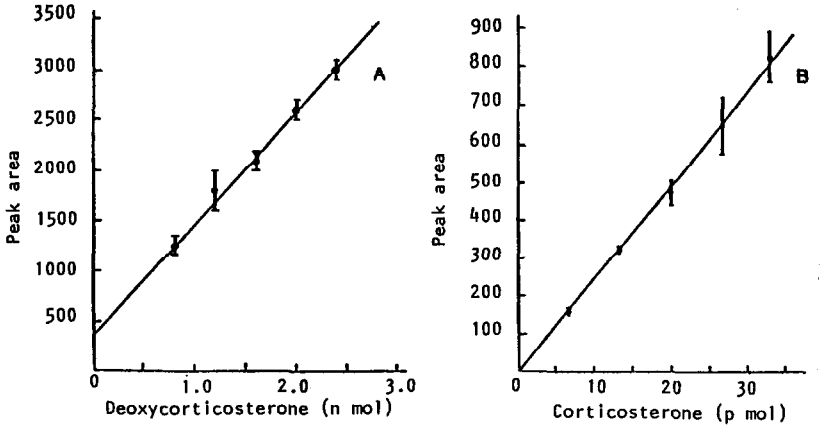

Fig. 2. Standard curves obtained by HPLC. (A) Deoxycorticosterone; (B) corticosterone. Each point represents at least three determinations. Error bars indicate confidence limits of 90%.

RESULTS

Separation of deoxycorticosterone and cortinosterone

The steroids were separated within 5 min, deoxycorticosterone having a retention time of 3 min and corticosterone 4.25 min (Fig. 1). Linear correlations were obtained between peak area and the amount of steroid injected (Fig. 2) and both

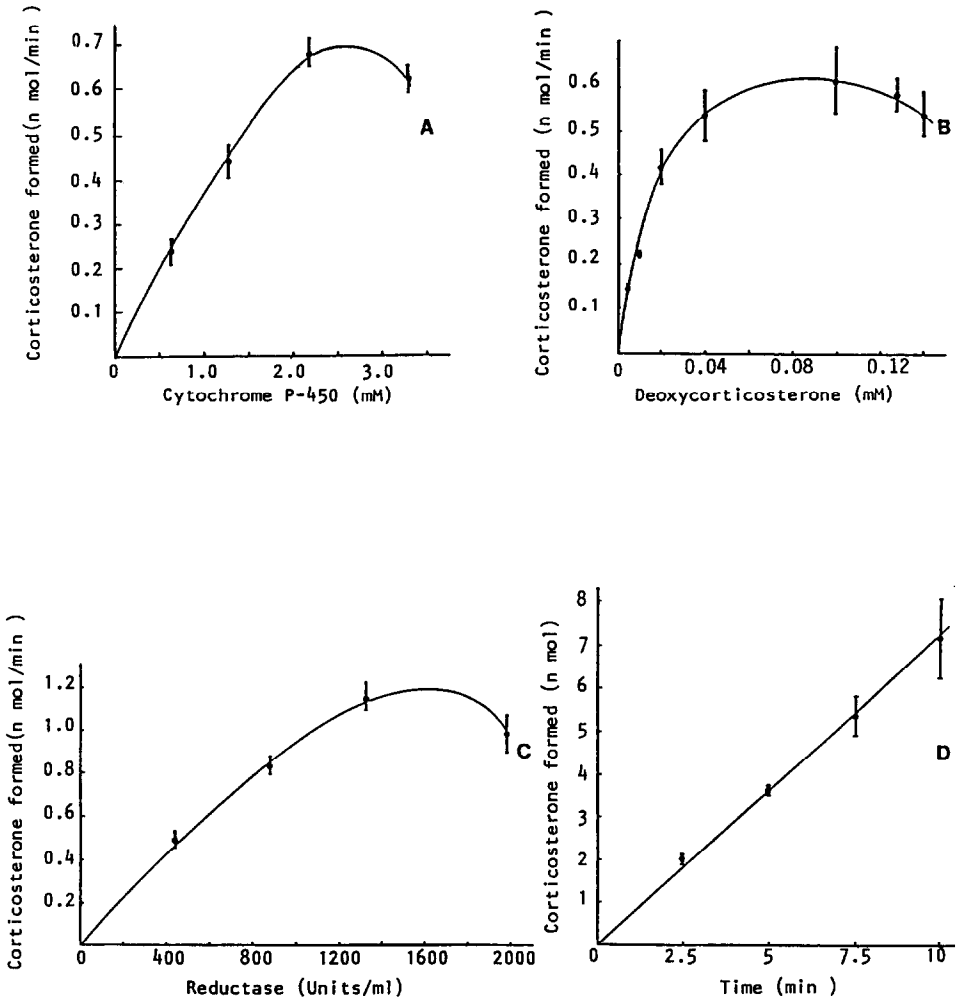

Fig. 3. (A) Corticosterone formation as a function of cytochrome P-450 concentration. Standard assay conditions were used. Cytochrome P-450 concentrations were 0.64, 1.28, 2.23 and 3.35 μM. Each point represents at least three determinations. Errors bars indicate confidence limits of 80%. (B) Corticosterone formation as a function of deoxycorticosterone concentration. Standard assay conditions were used. Deoxycorticosterone concentrations were 0.005, 0.10, 0.02, 0.04, 0.1, 0.124 and 0.14 mM. Each point represents at least three determinations. Error bars indicate confidence limits of 80%. (C) Corticosterone formation as a function of ADXR–ADX concentration. Standard assay conditions were used. Each point represents at least three determinations. Error bars indicate confidence limits of 80%. (D) Time course study of corticosterone formed under standard assay conditions. Each point represents at least three determinations. Error bars indicate confidence limits of 80%.

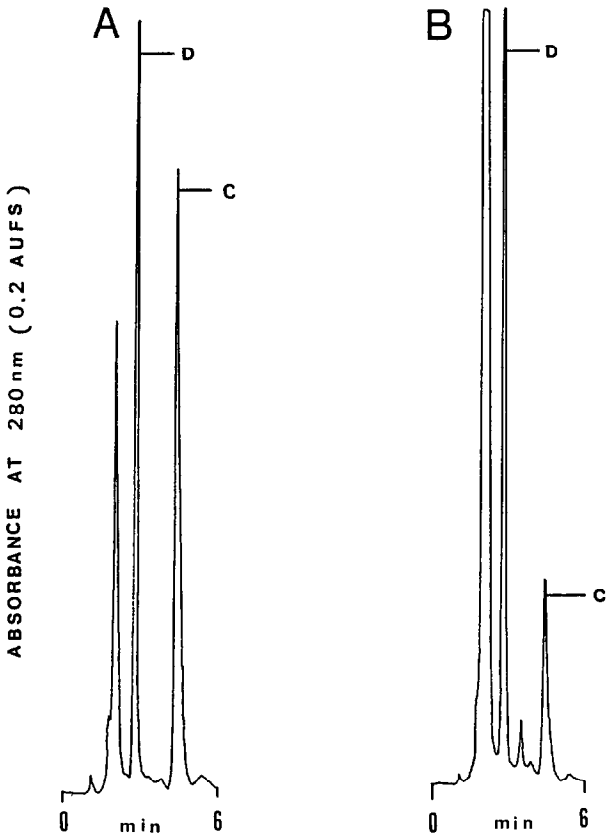

Fig. 4. Separation of deoxycorticosterone (D) and corticosterone (C) after extraction of steroids with dichloromethane. (A) Standard control incubation; (B) incubation containing an inhibitor.

steroids could be quantitated in the nanomole range. The compounds in the plant extract eluted as one single peak ahead of the steroids which were adequately resolved for accurate quantitation.

Conversion of deoxycorticosterone to corticosterone

Ideal assay conditiosns were obtained by determining the dependence of corticosterone formation on the concentration of cytochrome P-450, ADX–ADXR preparation and deoxycorticosterone as well as on time (Fig. 3A–D).

The separation of deoxycorticosterone and corticosterone after incubation and extraction in the absence and in the presence of an active inhibiting plant fraction is shown in Fig. 4A and B, respectively.

DISCUSSION

The multiple assays that had to be performed during our investigation of biologically active substances from *S. tuberculata* required a rapid, practical microassay

for sheep 11β-hydroxylase that could be carried out on a routine basis. The use of partially purified enzyme preparations in a total incubation volume of 200 μl madce it possible to develop such an assay. The substrate and product could be extracted from a complex reaction mixture and quantitated accurately by HPLC, indicating that this direct assay can be used to study the influence of the active compounds on corticosteroid production in while mitochondria or even adrenal cell cultures. Such information would be invaluable in determining the biochemical effects of natural inhibitors of adrenal steroid hormone biosynthesis.

The separation of all the major adrenal steroids by normal as well as reversed-phase HPLC have been previously reported by several workers in this field[10–13]. For this study an isocratic separation on a normal-phase column was developed that could separate the required steroids in less that 5 min, saving a considerable amount on analysis tiome and solvents. The one-step separation and quantitation of steroid substrate and product in the 11β-hydroxylase assay holds distinct advantages over methods previously published[3–7]. Small volumes of steroid solution could be separated by HPLC without interference by test compounds in less than 5 min. As separation and quantitation is done in one single step, sample handling is reduced to a minimum, eliminating the restrictions and precautions imposed by the use of radioactive materials. The use of an immunoassay was considered, but the possibility that the active compounds could be steroid analogues existed and therefore ruled out this method for steroid quantitation.

The interaction of a substrate or inhibitor with the active site of cytochrome P-450-dependent enzym,es may also be studied by difference spectroscopy[2]. These experiments only require the presence of the cytochrome P-450 moiety without any co-factors. Results obtained with the microassay described above were verified with spectral assays, using the well known inhibitor of 11β-hydroxylase activity, metyrapone, as a model compound compared to S2, a biologically active fraction isolated from *S. tuberculata* (see Fig. 5). The results of these experiments showed that the microassay was reliable and that it could be used to detect small amounts of inhibitor.

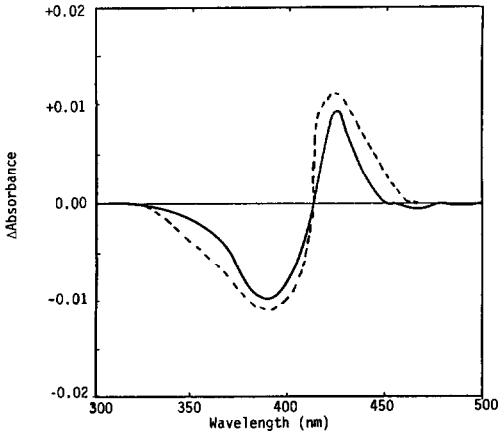

Fig. 5. The influence of metyrapone (solid line) and a fraction of plant extract (S2) from *S. tuberculata* (broken line) on the spectral properties of cytochrome P-450$_{11\beta}$.

Unlike the spectral assays, which only monitor the activity of the cytochrome P-450 component of the 11β-hydroxylase system, compounds acting on the co-factors, ADX and ADXR, can also be detected with this microconversion assay.

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