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Short Communication

Liquid chromatographic assay with ultraviolet detection for the study of microsomal steroid metabolism

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Introduction

Endogenous steroids are exposed to a number of cytochrome P-450 catalysed monooxygenase reactions in the endoplasmic reticulum of the adrenal glands and the liver. Whilst the reactions in the adrenals are synthetic in nature, hepatic steroidal metabolism is largely, catabolic (Fig. 1). These reactions involve highly stereo- and regio-specific isozymes, many of which have been extensively studied and characterized, e.g. P-450 IIIA4 and P-450 IIC11 which catalyse 6β and 16α-hydroxylations, respectively [1–2]. Other reactions that have been studied include the 2α, 5α, 6α, 6β, 15α and 16α-hydroxylations in the liver, and 17α-hydroxylase/lyase and C21-hydroxylase reactions in the adrenocortical microsomes [3–6].

To date, most investigators have employed either thin layer chromatography (TLC) [7], thin layer chromatography/gas chromatography mass spectrometry [4, 6, 8] or liquid chromatography (LC) [3, 9–11] for separation and quantitation of the products of microsomal or mitochondrial steroid metabolism. Whilst conventional TLC methods are relatively non-specific compared with recent HPLC methods, the latter usually involve the use of radiolabelled substrates [3, 12]. Where non-radiometric methods have been used, they have generally not attempted to exclude the co-elution of minor metabolites.

This is a report of our development of a sensitive and highly specific HPLC assay for a number of major as well as minor metabolites of progesterone metabolism. The method may be used for characterizing steroidal metabolism in liver and adrenal microsomes.

Experimental

Reagents

The following steroids: 2α, 6α, 6β, 15α, 16α and 17α-hydroxyprogesterone, androstenedione, deoxycorticosterone and deoxycortisol (used as authentic standards) and progesterone (used as substrate), were purchased from Steraloids (Wilton, NH, USA), with the exception of 15α-hydroxyprogesterone which was a generous gift from The UpJohn Co. (Kalamazoo, MI, USA). HPLC grade acetonitrile, as was analytical grade methylene chloride and methanol, were obtained from J.T. Baker (Phillipsberg, NJ, USA).

Sample preparation

Rat liver and pig adrenal microsomes were prepared by differential ultracentrifugation. Microsomal incubation, initiated by the addition of progesterone (100 μM) and NADPH (1 mg) was performed at 37°C for 2.5 min in a total volume of 1 ml. Following the addition of the internal standard, 11-hydroxytestosterone (1 μg), the steroids were extracted with 6 ml of methylene chloride. The organic phase was evaporated under a stream

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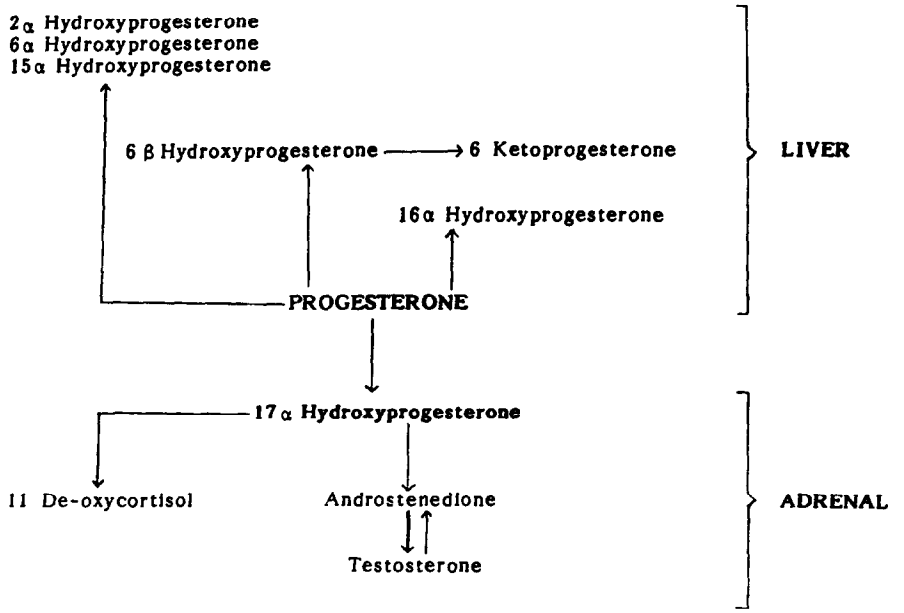

Figure 1
Cytochrome P-450 dependent metabolism of progesterone in liver and adrenal microsomes.

of nitrogen, after which the dried residue was reconstituted in 200 μl of acetonitrile, 20 μl of which was injected onto the LC column for analysis.

Liquid chromatography

Liquid chromatography was performed at ambient temperature using a Varian Vista system consisting of a pump with a ternary proportioning valve, a UV detector (Varian UV200), a data station (Varian Vista 402) and a systems controller. Separation of progesterone and its metabolites was achieved using two reversed-phase Hibar C18 5 μM (250 × 4.6 mm) columns connected in series. A 2 μM column inlet filter and a guard column (7.5 × 2.1 mm, Chrompak) packed with the same reversed-phase material was placed before the analytical column.

The mobile phase was an acetonitrile–water mixture, programmed to be delivered at a flow rate of 0.5 ml min^{-1} at the following gradient: 40:60; (initial) 60:40 at 20 min; 70:30 at 35 min; and 100:00 at 55 min. The column pressure generated was approximately 150 bar. The eluent was monitored at 240 nm.

Verification of the assay

Retention times of the various compounds were determined by injecting a methanolic solution (20 μl) containing 100 ng of each of the authentic standards. The identities of unknown peaks were confirmed by their respective retention times.

Calibration curves were obtained for each of the authentic standards by plotting their respective peak height ratio to the spiked concentration in microsomes (0.1–1.8 μg ml^{-1}). Metabolite concentrations after sample incubation were determined by interpolating their peak heights ratios on the respective calibration curves.

Steroidal standards were prepared using microsomes which had been pre-incubated in the absence of substrate and NADPH. Recoveries of progesterone and its metabolites using the extraction procedure described were determined by comparison of the peak height obtained from injection of the extracted standard to that of an equivalent amount of standard injected directly into the column.

Reproducibility of the assay was determined by measuring five aliquots of the steroid standards (0.1 to 1.0 μg ml^{-1}) in microsomes on the same day. An additional five aliquots were assayed over 5 consecutive days to derive measures of the day-to-day variability of the assay.

The accuracy of the assay was estimated by comparing the results obtained from assay of four aliquots of a microsomal standard to the actual steroid concentrations in the standard.

Results and Discussion

With the exception of 2α-hydroxy/6-keto-progesterone and 15α/17α-hydroxyprogesterone, the other steroids were well resolved from

Table 1
Retention times, extraction recoveries, accuracy (n = 4) and assay relative standard deviations (RSD, n = 5) of some progesterone metabolites

Compounds	Retention times (min)	Recovery (%)	RSD (%) Within-day	RSD (%) Inter-day	Accuracy (%)
Progesterone	52.14	103.47	4.5	2.0	100 ± 5
11β-OH Testosterone	20.92	80.35	1.7	3.6	99 ± 2
Hepatic metabolism					
15α-OH Progesterone	24.30	93.61	7.5	10.0	89 ± 10
16α-OH Progesterone	24.98	85.18	1.4	7.7	99 ± 1
6α-OH Progesterone	27.11	93.87	1.1	5.7	108 ± 10
6β-OH Progesterone	31.34	89.06	1.1	1.8	101 ± 1
6-Ketoprogesterone	35.93	89.54	3.9	5.2	99 ± 2
2α-OH Progesterone	35.95	70.36	5.2	5.2	104 ± 8
Adrenal metabolism					
11-Deoxycortisol	22.36	60.53	2.0	3.4	104 ± 6
17α-OH Progesterone	24.30	93.14	1.7	1.8	102 ± 1
Testosterone	34.74	82.52	4.1	4.5	104 ± 4
Androstenedione	36.92	71.20	1.28	5.4	100 ± 1
11-Deoxycorticosterone	45.74	96.50	5.03	3.87	96 ± 4

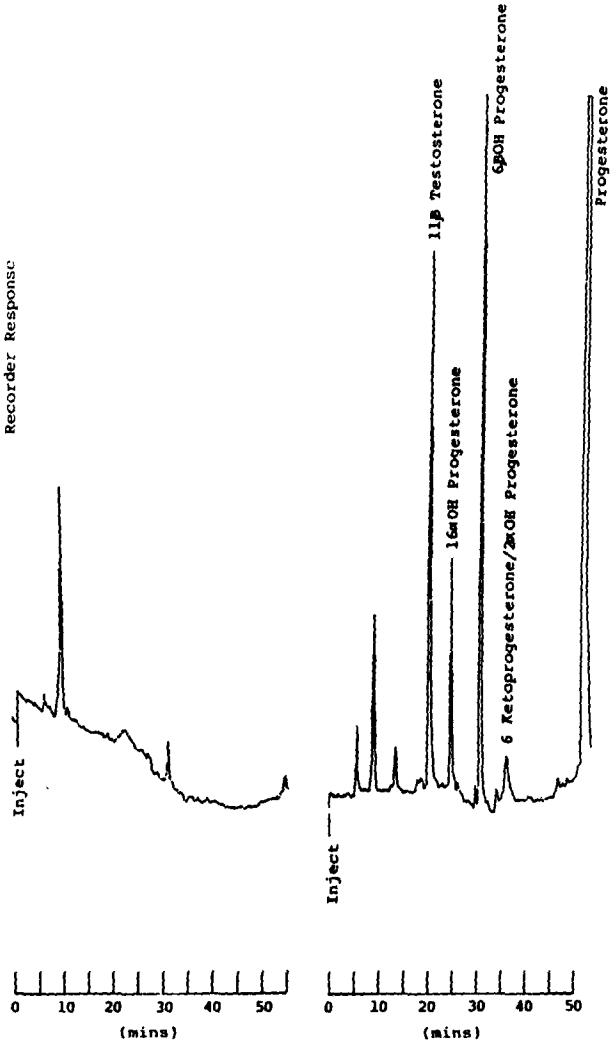

Figure 2
Typical chromatogram obtained following the incubation of rat liver microsomes, (a) without and (b) with 100 μM progesterone as substrate.

each other. The retention times of all the steroids assayed are given in Table 1. The co-elution of 2α-hydroxy/6-ketoprogesterone may be expected to limit the use of this method for the assay of these metabolites, however, these are relatively minor metabolites. On the other hand, as 15α and 17α-hydroxyprogesterone are generated in liver and adrenal microsomes, respectively, their co-elution under these conditions does not present a problem. The method may thus be used for their respective analysis. Figures 2 and 3 show typical chromatograms obtained after incubation of progesterone with pig adrenal and rat liver microsomes, respectively.

Recoveries of the steroids with the extraction procedure were consistently high for the various metabolites, and ranged between 60.53% for 11-deoxycortisol to 103.47% for progesterone (see Table 1).

Calibration curves were linear ($r2 < 0.99$) within the concentration range studied. The gradients and intercepts of the regressed lines for the respective standards were: progesterone (0.01986, 0.0309), deoxycorticosterone (0.0129, −0.0045), 11-deoxycortisol (0.0106,

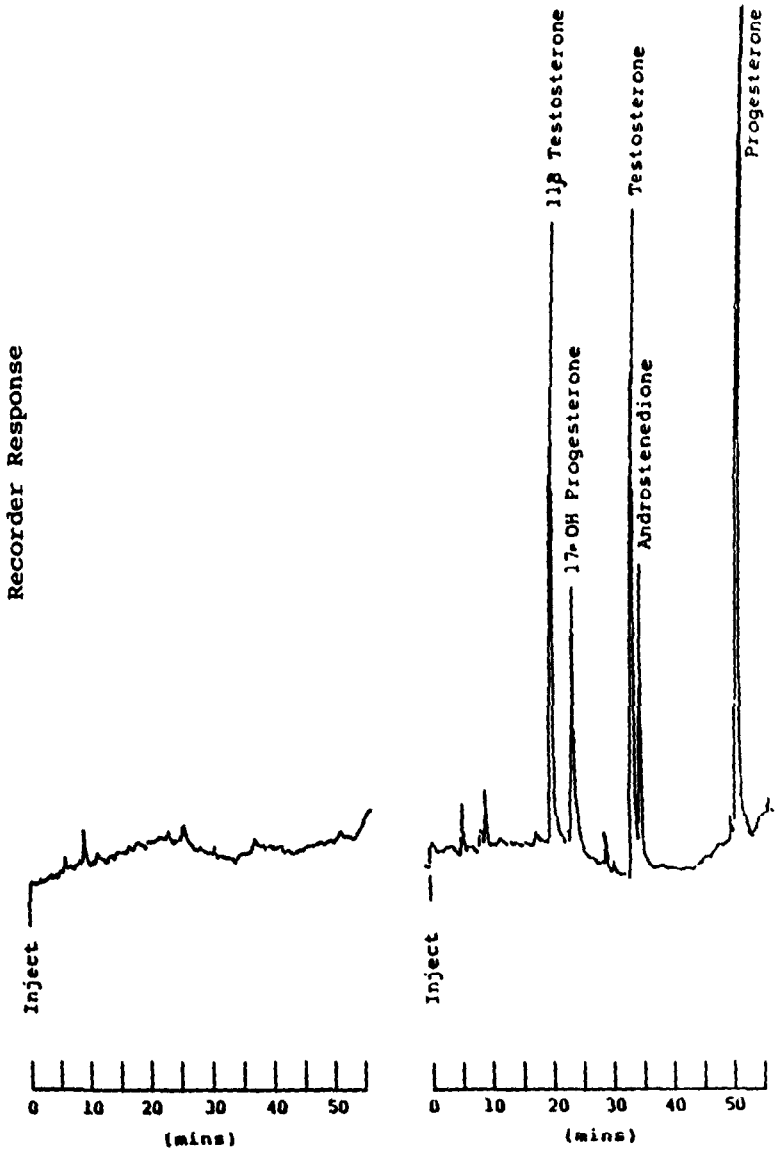

Figure 3
Typical chromatogram obtained following incubation of pig adrenal microsomes, (a) without and (b) with 100 μM progesterone as substrate.

−0.0031), testosterone (0.0349, 0.0817), androstenedione (0.0385, −0.2774), 6-keto-progesterone (0.0080, 0.0047), 6β-hydroxy-progesterone (0.0181, 0.0100), 2α-hydroxy-progesterone (0.0033, 0.0553), 6α-hydroxy-progesterone (0.0303, 0.0136), 15α-hydroxy-progesterone (0.0300, 0.0179) and 11β-hydroxytestosterone (0.0274, −0.1145). Accepting a signal–noise ratio of 4, the minimum detection amount of the steroids on the column was 10 ng.

The assay is highly reproducible, with a within-day relative standard deviation (RSD) of 1.1–7.5% and inter-day RSD of 1.8–10% (Table 1). Accuracy of the assay lies within the range 89–108%.

The LC method described is therefore relatively simple, with a high degree of sensitivity and specificity. It is easily automatable and may be effectively used for the characterization of steroidal metabolism in both adrenal and liver microsomes.

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