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Note

Separation of metabolites of testosterone hydroxylation by high-performance liquid chromatography

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The hepatic microsomal monooxygenase consisting of the proteins, cytochrome P-450 (cyt. P-450) and NADPH-cyt. P-450 reductase, oxidizes a variety of substrates including steroids, polynuclear aromatic hydrocarbons and a large number of drugs[1,2]. Several forms of cyt. P-450 from the rat[3], rabbit[4] and mouse[5] have been purified to apparent homogeneity and can be differentiated on the basis of their catalytic properties towards selected substrates in a reconstituted system. One substrate which has proved to be highly valuable in distinguishing the major forms of cyt. P-450 is testosterone. This steroid is hydroxylated at the 2β-, 6β-, 7β- and 16α-positions[6] and each of the last three metabolites is thought to be formed by a specific cyt. P-450[3,5,7]. The metabolism of testosterone therefore can be used to probe the major cyt. P-450s in microsomal preparations by determining the pattern of hydroxytestosterones.

The assay of the monooxygenase with testosterone as substrate is usually carried out with radioactively labelled substrate. A paper chromatographic method for the separation of testosterone and its 2β-, 6β-, 7α- and 16α-hydroxy metabolites has been reported[6]. This procedure is time-consuming and does not permit the separation of more polar metabolites. Recently, Shiverich and Neims[8] have reported a thin-layer chromatographic (TLC) method for 2β-, 6β-, 7α- and 16α-hydroxytestosterones, testosterone and androstenedione. It is an improvement over the paper chromatographic separation because it shortens the time of analysis.

My interest in the metabolism of testosterone by the hepatic microsomal monooxygenase and the need to simplify the analysis of the products have led to the development of a high-performance liquid chromatographic (HPLC) method for the separation of 2β-, 6β-, 7α- and 16α-hydroxytestosterones, testosterone and androstenedione. The main advantages of HPLC over TLC and paper chromatography are its speed of analysis, selectivity and sensitivity and no radioactively-labeled steroid is needed. In addition, it permits the detection of metabolites other than those referred to above.

MATERIALS AND METHODS

The HPLC system consisted of a high-pressure minipump (Milton Roy,

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Riviera Beach, FL, U.S.A.), a Model 1840 variable-wavelength absorbance monitor (ISCO, Lincoln, NE, U.S.A.), a 25 cm × 4.6 mm I.D. 10-μm C_{18} reversed-phase column preceded by a guard column, a sample injection valve and pressure gauge (Alltech Assoc., Arlington Heights, IL, U.S.A.). The absorbance of the effluent was recorded at 240 nm on a Recordall Model 5000 recorder (Fisher Scientific, Rochester, NY, U.S.A.). Separation was carried out at ambient temperature at a flow-rate of 0.97 ml min^{-1} and 2100 p.s.i. with a mobile phase of methanol–water (55:45), followed by methanol–water–acetonitrile (55:35:10) at 23 min. Recrystallized testosterone (4-androsten-17β-ol-3-one, T) was a gift from Dr. Frederick H. Batzold of the Department of Pharmacology and Experimental Therapeutics of the Albany Medical College. 4-Androstene-2,17β-diol-3-one (2β-hydroxytestosterone, 2β), 4-androstene-6,17β-diol-3-one (6β-hydroxytestosterone, 6β), 4-androstene-7α,17β-diol-3-one (7α-hydroxytestosterone, 7α) and 4-androstene-16α,17β-diol-3-one (16α-hydroxytestosterone, 16α) were obtained through the courtesy of Steroid Reference Collection, MRC, London. 4-Androstene-3,17-dione (androstenedione, A) was purchased from Sigma (St. Louis, MO, U.S.A.). All steroids were dissolved in methanol. Methanol and acetonitrile were purchased from VWR (Boston, MA, U.S.A.) and water was double-glass-distilled.

RESULTS AND DISCUSSION

The five major products of testosterone formed by the monooxygenase can be separated by HPLC as shown in Fig. 1. 16α- and 2β-Hydroxytestosterone were well resolved from the other components. No baseline separation of 7α- and 6β-hydroxytestosterone could be achieved, however, with the mobile phase described above. Decreasing the ratio of methanol to water did not improve markedly the resolution of these two metabolites. Acetonitrile has been used for the separation of steroid hormones by O'Hare *et al.*[9]. We observed that CH_3CN–H_2O (40:60) separated all the steroids except 6β- and 16α-hydroxytestosterone. The same system eluted 2β-hydroxytestosterone as a broad band. Because of the shorter analysis time with the acetonitrile–water system a number of organic solvent modifiers were added to improve the resolution. In general, the addition of tetrahydrofuran or 2-propanol decreased, and of glacial acetic acid or methanol increased, the elution time of the metabolites without improvement in the separation of 6β- and 16α-hydroxytestosterone. The separation of testosterone, 7α- and 16α-hydroxytestosterone by reversed-phase (C_{18}) chromatography with CH_3CN–H_2O gradient elution has been reported[9]. However, it is not clear from the data whether a baseline separation of 7α- and 16α-hydroxytestosterone was achieved. The elution times of the 7α- and 16α-hydroxy metabolites were 9 and 11 min, respectively. A much larger difference in elution time of these two metabolites was obtained with a methanol–water gradient[9]. A separation of 6β-hydroxytestosterone and testosterone was observed by Hara and Hayashi[10] on a silica column with *n*-hexane and ethyl acetate. The major advantage of reversed over normal phase chromatography in drug metabolic studies is the order of elution of the analyte; the less polar are retained longer than the more polar compounds. Since the oxidation products are more polar than the percursor, the hydroxylated metabolites of testosterone are eluted earlier than testosterone in reversed-phase chromatography. In incubation mixtures the concentration of testosterone is very

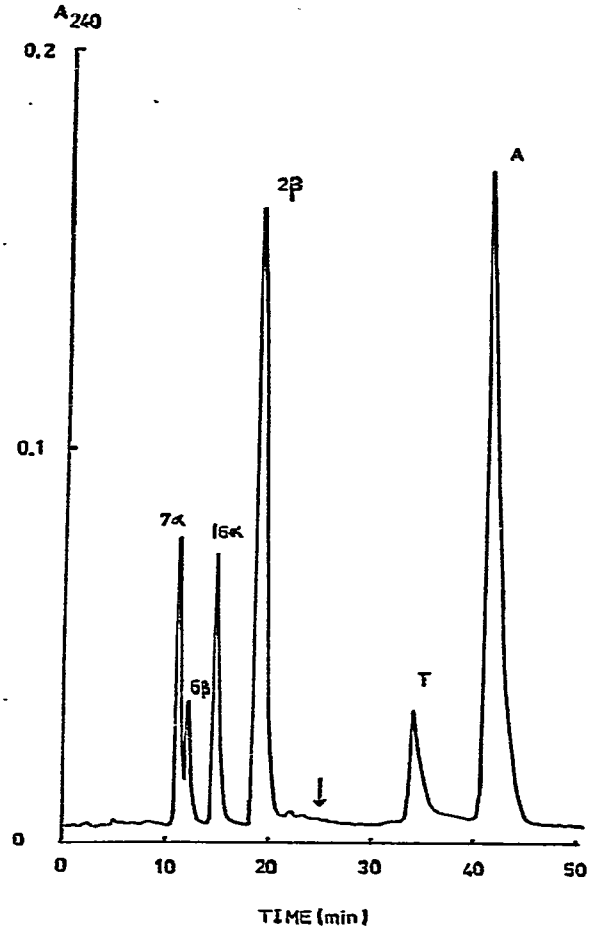

Fig. 1. Separation of hydroxylated testosterones, testosterone and androstenedione by reversed-phase column chromatography under conditions as described in the text. The injected sample volume is 10 μl and the concentration of each steroid is: 2β-hydroxytestosterone (2β-), 1.32 m*M*, 6β-hydroxytestosterone (6β-) 0.33 m*M*, 7α-hydroxytestosterone (7α-) 0.33 m*M*, 16α-hydroxytestosterone (16α-) 0.37 m*M*, testosterone (T) 0.33 m*M* and androstenedione (A) 1.63 m*M*. The column was developed with methanol–water (55:45) followed by methanol–water–acetonitrile (55:35:10) initiated at the arrow.

much larger than that of the hydroxylated products and the earlier elution of the metabolites precludes the possibility of being obscured by the precursor. The greater retention of androstenedione as compared to that of testosterone has also been observed by O'Hare *et al.*[9].

The separation of testosterone metabolites was performed with a reversed-phase column purchased from one commercial company. Differences in the selectivity of C_{18} reversed-phase columns from several commercial sources have been noted[11]. Greater or poorer resolution may be encountered with columns from other manufacturers. Nice and O'Hare[12] observed marked differences in the retention of steroid hormones with C_{18} reversed-phase columns from five suppliers, and suggested that

these differences in selectivity can be taken advantage of to achieve a particular analysis.

In summary, a HPLC method is described for the separation of the major hydroxylated metabolites of testosterone by the hepatic microsomal monooxygenase. In preliminary studies the method was found to be sufficiently sensitive for the quantitation of the products of testosterone formed upon incubation of the steroid with rat liver microsomal fraction.

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