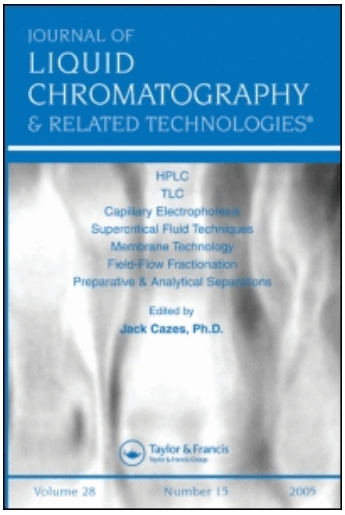

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Determination of Hydroxy-Testos-Terones by Isocratic, High-Performance Liquid Chromatography with an Internal Standard

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DETERMINATION OF HYDROXY-TESTOSTERONES BY ISOCRATIC, HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH AN INTERNAL STANDARD

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ABSTRACT

A simple, isocratic, reverse-phase HPLC method to separate nine oxidative metabolites of testosterone is described. An internal standard, adrenosterone, was used to quantitate metabolites by the peak height method. An application of this method to the metabolism of testosterone by rat lung microsomes is presented.

INTRODUCTION

The profile of testosterone metabolites formed by both intact and reconstituted microsomes is a useful tool to characterize individual isozymes of cytochrome P-450 in liver (1,2). Since the apparent Km values for testosterone hydroxylation are about 10-fold lower than the Km values for the metabolism of most drugs (3), and several metabolites are generated from a single aliquot of protein, such an approach would be particularly useful to study cytochrome P-

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450 in extrahepatic tissues where the yield of microsomal protein and enzyme activity are generally low (4).

Early studies of testosterone hydroxylation by liver microsomes utilized paper (5) and thin-layer (6) chromatography for metabolite quantification. However, these methods do not adequately resolve all metabolites, require radiolabelled substrate, are time consuming, and may lack sensitivity depending on the relative amounts of metabolites formed.

The generation of small amounts of metabolites by extrahepatic tissues requires a sensitive method, with good resolution. Several HPLC methods that measure hydroxy-testosterones have been described, but they generally require gradient elution (7,8), have a long separation time (9,10), or are capable of resolving only a few metabolites (11). Therefore, we developed a simple, isocratic HPLC method with internal standardization to quantitate nine oxidative metabolites of testosterone.

EXPERIMENTAL

Standards

Testosterone and 16-keto-testosterone were obtained from Sigma Chemical Co. (St. Louis, MO). Adrenosterone, 7α-, 16α-, and 16β-hydroxy-testosterone were purchased from Steraloids, Inc. (Wilton, NH). The remaining metabolites (6α-, 15α-, 6β-, 2α-, and 2β-hydroxy-testosterone) were kindly provided from the Steroid Reference Collection of the Medical Research Council, London, United Kingdom, by Dr. N. Kirk via Dr. D.F. Johnson of the NIH.

Sample Preparation

Metabolite standards, ranging from 0.1 to 5.0 nmol, were added to inactivated microsomes (1 mg protein per ml of 0.1 M HEPES buffer, pH 7.55). Internal standard, 500 ng adrenosterone (4-androstene-3,11,17-trione), was added to all lung incubations and microsomes spiked with calibration standards. The steroids were extracted into 5 ml methylene chloride which was subsequently evaporated to dryness

at 45°C under nitrogen. The residue was dissolved in 0.5 ml methanol which was also evaporated at 60°C under nitrogen. The residue was then redissolved in 20µl methanol for injection onto the HPLC.

Apparatus

The HPLC consisted of 2 model 510 pumps, model 481 UV detector, and model U6K injector (Waters, Inc., Milford, MA). Samples were chromatographed on a Zorbax ODS (25 cm x 4.6 mm) column (Du Pont Co., Wilmington, DE) fitted with a 2 cm, 5 µ, LC-18 guard column (Supelco, Bellefonte, PA). The mobile phase was A/B (90:10) where A = water:methanol:acetonitrile (46:30:24) and B = water:methanol (8:92) at a flow rate of 0.8 ml/min. To speed the elution of less polar metabolites, the flow rate was increased to 1.4 ml/min at 16 min. Amounts of the metabolites in samples were extrapolated from calibration curves that were constructed by plotting the peak height ratios of individual reference standards to internal standard vs the amount of standard.

RESULTS AND DISCUSSION

Typical chromatograms of metabolite standards and metabolites formed by rat lung microsomes are shown in figure 1A and B, respectively. Near baseline resolution was achieved for 6 metabolites, 6α-, 15α-, 7α-, 6β-, and 16α-hydroxy-, and 16-keto-testosterone. Separation of 16β-, 2α-, and 2β-hydroxy-testosterone was incomplete. Tredger *et al.* (10) were also unable to resolve these three metabolites. However, as seen in figure 1B, the amounts of 16β- and 2β-hydroxy-testosterone formed by rat lung were completely resolved in this system. In contrast to previously published methods (8,10), 15α-hydroxy-testosterone was eluted as a discrete peak under our chromatograpic conditions.

Not shown in figure 1 is the elution of testosterone and less polar metabolites, such as androstenedione. By flow-rate programming, we were able to elute testosterone at 25.4 min and all peaks were eluted by 30 min. This is in sharp contrast with the total run time required for other isocratic (10) or gradient methods (8).

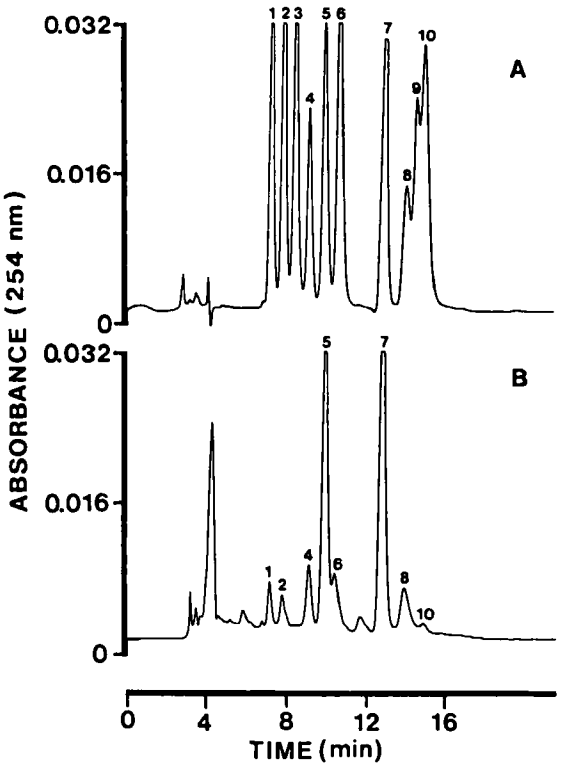

FIGURE 1. Representative chromatograms of testosterone metabolites. 1 = 6α-OH-T, 2 = 15α-OH-T, 3 = 7α-OH-T, 4 = 6β-OH-T, 5 = 16α-OH-T, 6 = 16-keto-T, 7 = Adrenosterone (internal standard), 8 = 16β-OH-T, 9 = 2α-OH-T, 10 = 2β-OH-T.
A) Mixture of metabolite standards
B) Profile of metabolites formed by rat lung microsomes.

Adrenosterone was chosen as an internal standard because it is not generated by microsomal incubations, and, unlike other internal standards that have been used, such as 11β-hydroxy-testosterone (9), is completely resolved from all metabolite peaks.

Representative plots of peak height ratio vs amount of standard are shown in figure 2. Linearity of response was achieved from 0.1 through 5.0 nmol of each metabolite. Calibration curves for the other metabolites were similar with peak height ratios decreasing as

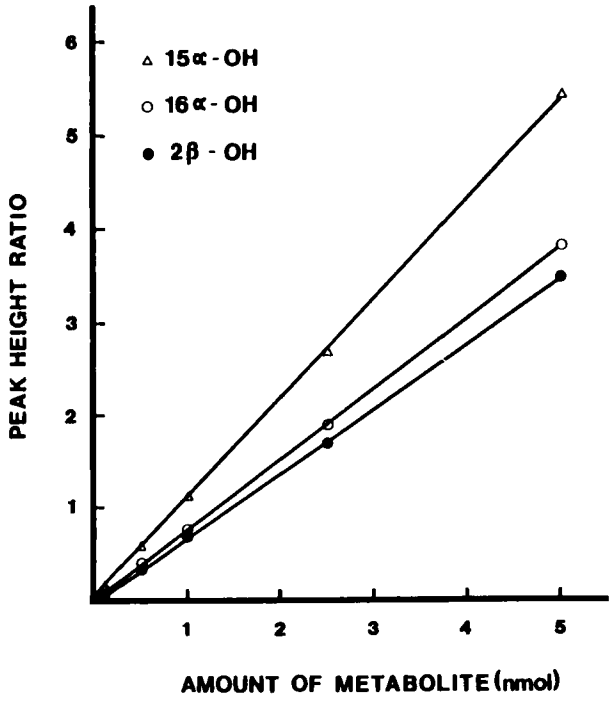

FIGURE 2. Calibration curves for selected metabolites of testosterone. Each point is the mean of 10-12 determinations.

a function of increased retention time as would be expected. The standard deviation for 10-12 determinations at 1.0 nmol was generally 5-7% of the mean.

Preliminary experiments with radiolabeled substrate yielded greater than 95% extraction of steroid from the microsomes. Therefore, individual recoveries of metabolites were not determined.

In conclusion, a simple HPLC method for the separation and quantitation of hydroxy-testosterones has been described. The main advantages of this method include a single set of mobile phase conditions, short separation time, and good resolution of even small amounts of metabolites. In addition, the use of an internal standard makes the method insensitive to variable recovery or partial loss of sample.

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