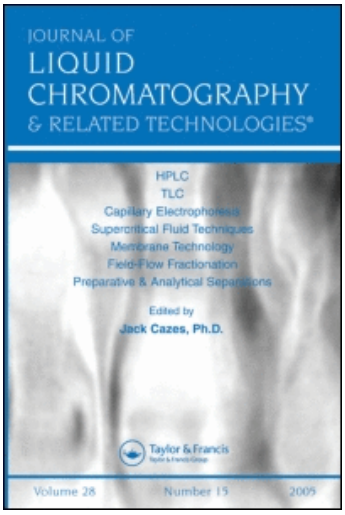

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Rapid Separation of Testosterone and its Microsomal Metabolites by Reverse-Phase High Performance Liquid Chromatography

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RAPID SEPARATION OF TESTOSTERONE AND ITS MICROSOMAL METABOLITES BY REVERSE-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

High performance liquid chromatography has been used to separate testosterone from its oxidative metabolites (7α-, 16α- and 6 β-hydroxytestosterone and androstenedione) following in vitro incubation with rat liver microsomes. The separation was accomplished in less than 18 minutes on a radially compressed C_{18} reverse-phase column using isocratic elution with tetrahydrofuran:water (24/76, v/v).

INTRODUCTION

Testosterone is an endogenous substrate for hepatic microsomal monooxygenases (1,2). This steroid is metabolized by rat hepatic microsomes to products such as 7α-, 16α- and 6 β-hydroxytestosterone and androstenedione in reactions that appear to require different forms of cytochrome P-450 (3). The hydroxylation of testosterone has been utilized in the characterization of several forms of purified hepatic cytochrome P-450 (4-6). Therefore, testosterone may be utilized as a single substrate

to quantify simultaneously the activity of several different enzymes present within a microsomal incubation mixture. Enzymes catalyzing specific hydroxylations of testosterone have been induced selectively by agents such as phenobarbital and 3-methylcholanthrene (7). A rapid and sensitive method for quantitation of testosterone and its major microsomal metabolites could be employed in the study of the effects of inducers of microsomal monooxygenases, such as the environmental contaminants polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs), on the microsomal metabolism of testosterone.

Several different methods have been developed for the analysis of testosterone and its metabolites. Descending paper chromatography has been the analytical method of choice for several investigators (3,6,7). However, a major disadvantage of such a technique is the relatively long sample development time and the necessity for radiolabeled samples. In addition, the complete resolution of the 4 major microsomal metabolites and the parent compound cannot be accomplished in a single development. Recently, thin-layer chromatographic methods have been reported which can separate all metabolites in a single development (5,8). However, a radiolabeled substrate of high specific activity is required for quantitation with this method. Recently, high performance liquid chromatography (HPLC) has been used in the analysis of testosterone and its metabolites. Shaikh et al. (8) reported a normal phase system that requires a long elution time and lacks a high degree of sensitivity. The reverse phase system reported by van der Hoeven (9) requires gradient elution and does not adequately separate the 7α- and 6β-hydroxy metabolites. Therefore, our goal was to develop a highly sensitive rapid isocratic HPLC system for analysis of testosterone and the four major microsomal metabolites.

MATERIALS AND METHODS

Microsomal Incubations and Sample Preparation

Testosterone and cofactors (Steraloids, Inc., Wilton, NH; Sigma Chemical Co., St. Louis, MO) were added in 66 mM Tris-HCl, pH 7.4, to

open screw cap test tubes to give a final volume of 1.06 ml. The reaction mixture contained approximately 1.15 μ mole testosterone, 0.3 μ mol NADH, 0.4 μmol NADP, 0.3 μmol NADPH, 5.8 μ mol glucose-6- phosphate, 1 unit of glucose-6-phosphate dehydrogenase, 3.0 μmol $MgCl_2$ and 0.8 mg microsomal protein. Following 1 min of preincubation, reactions were initiated by the addition of testosterone in 11 μ l of dimethyl sulfoxide. Reaction mixtures were incubated for 0-120 min, usually 30 min, at 37°C in a Dubnoff metabolic shaker. Reactions were terminated by addition of 5 ml of diethyl ether/chloroform (3:1, v/v). Incubation blanks were prepared by addition of diethyl ether/chloroform at zero time.

The reaction products were prepared for chromatography using the following method: samples were extracted with 3x5 ml of diethyl ether/chloroform (3:1, v/v); the organic fraction was dried by elution through anhydrous sodium sulfate, evaporated to dryness under N_2 and reconstituted in 1.0 ml of 95% ethanol for chromatography.

High Performance Liquid Chromatography (HPLC)

The HPLC system consisted of a Waters model M6000-A pump (Milford, MA) with a Model 440 uv detector set at 254 nm and a Model U6K injector. Samples were chromatographed on a Waters radial compression module with a 10 μm radial pak reverse phase (C_{18}) cartridge (8 mm x 10 cm) at ambient temperature. The solvent was tetrahydrofuran (THF):H_2O (24:76, v/v). The flow rate was maintained at 3.2 ml/min with a resulting column pressure of 800 psi. Peak areas were quantified using an electronic integrator (Shimadzu Seisakusho Ltd., Kyoto, Japan). Compounds were identified by their retention times relative to reference standards (Steraloids, Inc., Wilton, NH, and Medical Research Council, Steroid Reference Collection).

RESULTS AND DISCUSSION

Baseline separation of testosterone and the four major microsomal metabolites (7α , 16α and 6β -hydroxytestosterone and androstenedione) was

achieved with the THF-H_2O solvent system employed in this study. Interfering peaks were not present in the microsomal sample matrix as is evident from the chromatograph of the blank incubation (Fig. 1). Analysis of a single sample required less than 18 min; testosterone was the latest eluting peak in the chromatogram. Rat hepatic microsomes produced significant quantities of all four metabolites (Fig. 2). In addition hepatic microsomes from animals pretreated with PBBs (100 ppms) produced substantially more of the hydroxylated metabolites as well as several other

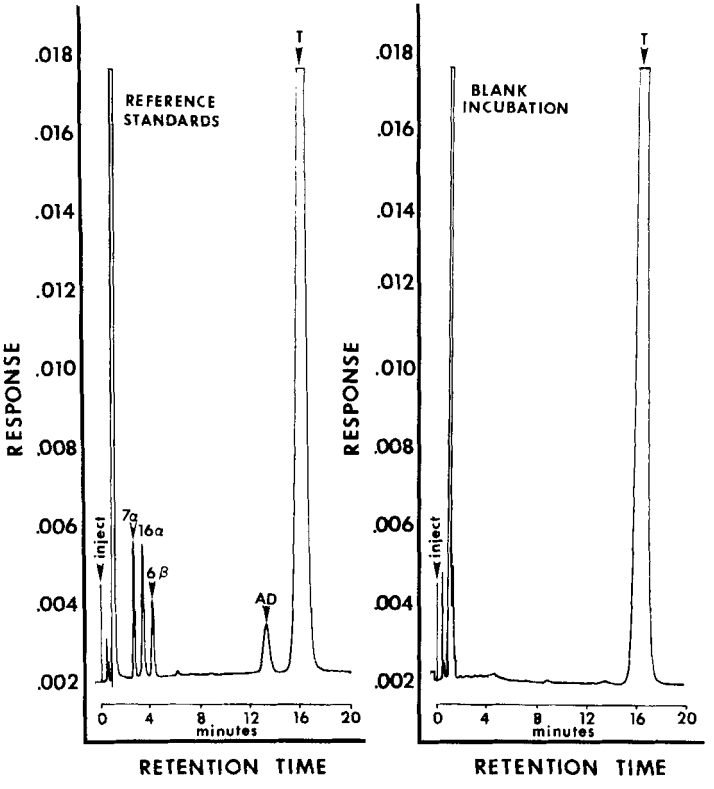

Fig. 1. HPLC profile of mixture of reference standards (7α-, 16α-, 6 β-hydroxytestosterone, androstenedione, and testosterone, left to right) and a blank incubation. An isocratic elution with THF/H_2O (24:76 v/v) was performed at a flow rate of 3.2 ml/min. UV absorbance was monitored at 254 nm at a full scale sensitivity of 0.02 absorbance units.

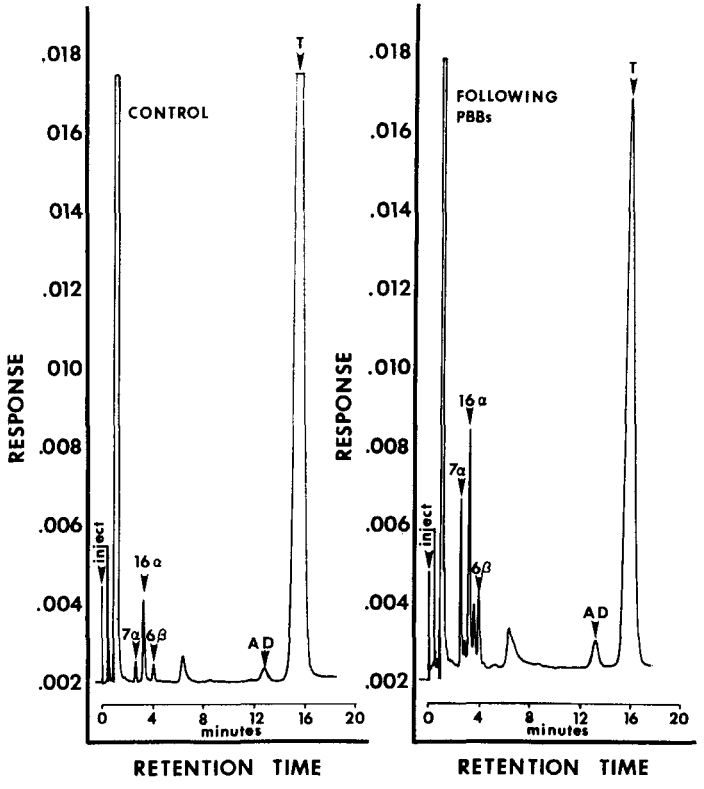

Fig. 2. HPLC profiles of microsomal incubations from untreated and PBB treated rats. HPLC conditions were the same as those described in the legend for Fig. 1.

unidentified peaks (9). However, even in the presence of these peaks, resolution was sufficient to allow quantitation of all three of the hydroxylated metabolites (Fig. 2).

Gas chromatography-mass spectrometry (GC/MS) of the methoxime-trimethylsilyl ethers derivative of HPLC peaks from actual incubation samples revealed that the major component of each HPLC peak had retention time and mass spectrum identical to the corresponding reference standard for the metabolite in question. No additional steroidal compo-

nents were evident by GC/MS analysis of the isolated HPLC peaks. Quantitation of testosterone and metabolites was linear over the range of values encountered in microsomal incubation mixtures. Amounts as low as 10 nanograms of steroid were easily quantified (Fig. 3).

A major advantage of the use of reverse phase chromatography was the early elution of all the polar microsomal metabolites. This provided much greater sensitivity than previously reported normal phase systems. In

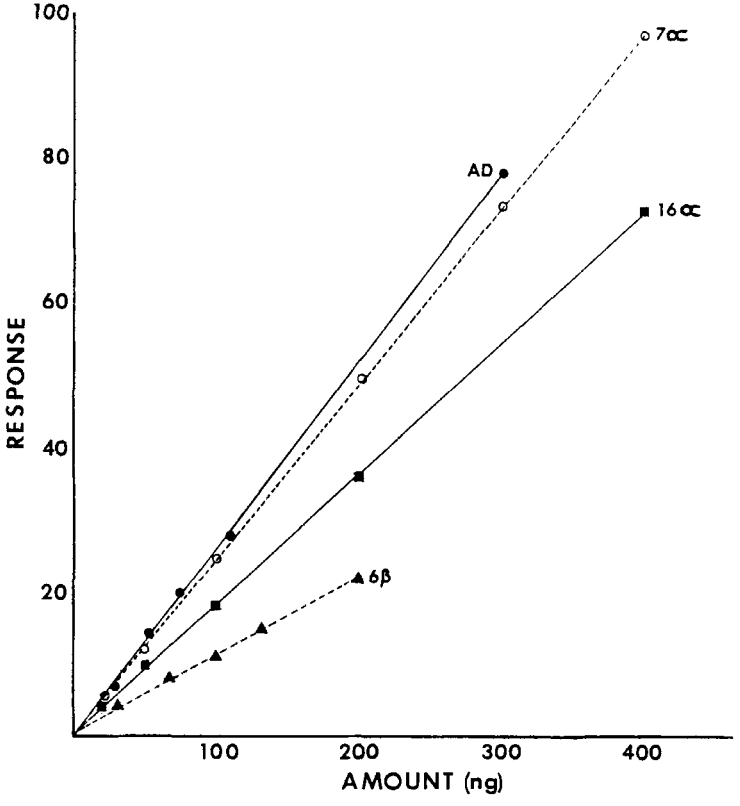

Fig. 3. Calibration curve for the four microsomal metabolites of testosterone expressed as peak area counts in mV·sec (response) as a function of varying amounts of metabolite. This curve covered the range of values encountered in the samples. HPLC conditions were the same as those described in the legend for Fig. 1.

addition, the late elution of testosterone in our system reduced the possibility that the metabolites may be obscured by the precursor in samples from a microsomal incubation mixture. Both acetonitrile and methanol have been used without success in the separation of all of the hydroxylated metabolites of testosterone (10). However, the separation of 7α- and 16α-hydroxytestosterone, androstenedione and testosterone by reverse-phase HPLC utilizing dioxane:water solvent gradient has been reported (11). Therefore, it is not surprising that another ether, tetrahydrofuran, provided baseline separation of all metabolites studied. Perhaps hydrogen bonding could have accounted for the selectivity gained by the use of THF. The rapid analysis time was an advantage gained by the high flow rates attainable with radial compression chromatography.

An HPLC method is now available for the rapid separation of the major hydroxylated metabolites of testosterone produced by rat hepatic microsomes. This method provides sufficient sensitivity for quantitation of these steroids in microsomal incubation mixtures containing less than one milligram of protein and incubated for less than 10 min under substrate saturated conditions.

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