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Measurement of enzyme activities of cytochrome P-450 isoenzymes by high-performance liquid chromatographic analysis of products

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ABSTRACT

A method is described for the qualitative and quantitative determination of the isoenzymes of cytochrome P-450 from rat liver microsomes. Microsomes are incubated with the endogenous steroid 17β-testosterone, which results in the formation of a number of stereo-specific hydroxylation products of testosterone. The hydroxylated products were identified using standards or by comparison with data from the literature. The products can be analysed by reversed-phase gradient high-performance liquid chromatography. The assay has been optimised for pH, linearity and time of incubation. An evaluation of the assay was performed for different kinds of microsomes, microsomal dilution and specificity for particular cytochrome P-450 isoenzymes.

INTRODUCTION

The cytochrome P-450 system consists of a number of isoenzymes which can be induced as a function of the presence of xenobiotic compounds. Each isoenzyme has its own specific function in the metabolism of exogenous and also endogenous compounds [1]. One of the enzymatic reactions which can be performed by cytochrome P-450 are hydroxylations of compounds to increase the polarity or to create possible sites for conjugation [2]. In addition to this detoxifying mechanism, unwanted processes may also occur. Xenobiotic compounds can be activated to reactive metabolites which can be covalently attached to proteins and nucleic acids. In biochemical toxicology the knowledge of the isoenzyme pattern of cytochrome P-450 is an important parameter in the study of organ toxicity. Methods of analysis such as chemiluminescence detection [3] or electrophoretic separation followed by immunochemical detection [4,5] can give information about the isoenzyme pattern. These techniques, however, do not necessarily reflect the real enzyme activity, because only the amount of protein is determined, which is not enzymatically active. Therefore methods are required which can give information about the real enzymatic activity of the individual isoenzymes. Well known specific enzymatic determinations are the

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fluorimetric assays using alkylcoumarins or alkylresorufins as substrates [6]. Several studies have been reported which use steroids as substrates, such as testosterone [7], androstenedione [8], progesterone [9], cholesterol [10] and pregnenolone [11]. In this laboratory a study has been initiated to develop a method in which all known cytochrome P-450 isoenzymes of the rat [12] can be quantitated, based on their enzymatic activity. This paper describes the first part of this study, which is the metabolic activity of cytochrome P-450 isoenzymes towards 17β-testosterone as the substrate.

EXPERIMENTAL

Chemicals

Testosterone and androstenedione were obtained from Sigma, Brunschwig Chemie (Amsterdam, The Netherlands). 6β-, 7α-, 11α-, 11β-, 16α-, 16β- and 19-hydroxytestosterone (OH-T) were from Steraloids, Brunschwig Chemie (Amsterdam, The Netherlands). All other chemicals used were of the highest analytical grade.

High-performance liquid chromatography equipment

The high-performance liquid chromatography (HPLC) equipment consisted of the following components: an automatic injector (Gilson, Model 231), two solvent delivery systems (LKB, Model 2150) controlled by a gradient controller (LKB, Model 2152), a variable-wavelength monitor (LKB, Model 2141) and a computing integration system (LDC/Milton Roy, Model CI-10B). The column used was a Chromsep C_{18} (200 × 3 mm I.D.) with 5 μm particle size (Chrompack, Middelburg, The Netherlands) preceded by a 10 mm × 3 mm I.D. C_{18} guard column (Chrompack).

Preparation of microsomes

Phenobarbital- and β-naphthoflavone-induced male rats. Livers were perfused with ice-cold saline, isolated, weighed and homogenised in ice-cold Tris–potassium chloride using a Potter–Elvehjem glass–PTFE homogeniser. The cell debris, nuclei and mitochondria were removed by centrifugation for 15 min at 16 000 *g* in a Sorvall centrifuge. The supernatant was centrifuged for 60 min at 105 000 *g* in a Centrikon ultracentrifuge. The pellet obtained at this centrifugation step was resuspended in Tris–potassium chloride buffer and again centrifuged for 60 min at 105 000 *g*. The microsomal pellet thus obtained was resuspended in Tris–sucrose buffer containing 25% glycerol, then quickly frozen in liquid nitrogen and stored at −70°C. The cytochrome P-450 content was determined spectrophotometrically according to the method of Omura and Sato [13]. The microsomes contained 1637 and 1290 pmol of cytochrome P-450 per mg of protein for the phenobarbital- and β-naphthoflavone-treated rats, respectively. Protein concentrations were determined according to the method described by Lowry *et al.* [14].

Control and the dexamethasone-induced rats. Livers were perfused with ice-cold saline, isolated, weighed and homogenised in ice-cold phosphate buffer with 1.15% potassium chloride using a Potter–Elvehjem glass–PTFE homogeniser. The cell debris, nuclei and mitochondria were removed by centrifugation for 10 min at 15 000 *g* in a Sorvall centrifuge. The supernatant was centrifuged for 30 min at 150 000 *g* in a Centrikon ultracentrifuge. The microsomal pellet obtained at this step was

resuspended in Tris–sucrose buffer containing 25% glycerol, then quickly frozen in liquid nitrogen and stored at −70°C. The cytochrome P-450 content was determined spectrophotometrically according to the method of Omura and Sato [13]. The microsomes of control rats contained 732 pmol of cytochrome P-450 per mg of protein (male rats), 655 pmol of cytochrome P-450 per mg of protein (female rats) and 1448 pmol of cytochrome P-450 per mg of protein for the dexamethasone-induced male rats. Protein concentrations were determined according to the method described by Lowry *et al.* [14].

In vitro assay

The procedure followed was that of Sonderfan *et al.* [15] with some modifications. The incubation mixture consisted of the following components: potassium phosphate buffer (50 m*M*, pH 7.4), which contained 1 m*M* EDTA, 3 m*M* magnesium chloride, 1 m*M* nicotinamide-adenine dinucleotide phosphate (NADP), 5 m*M* glucose-6-phosphate, 1 U/ml glucose-6-phosphate dehydrogenase, the steroid as substrate (250 μM, final concentration) and 40 μl of microsomes in a total volume of 1 ml. The reaction was started by the addition of the microsomes. After an incubation period of 15 min at 37°C the reaction was stopped by placing the mixture in ice-cold water followed by the addition of 6 ml of dichloromethane. At this point 11β-OH-T was added as an internal standard. After mixing and centrifugation the organic layer containing the metabolites was evaporated under a stream of nitrogen at room temperature. The residue was dissolved in 200 μl of methanol–water (50:50, v/v) for analysis by HPLC.

High-performance liquid chromatographic analysis

The elution conditions were as follows: isocratic elution for 10 min with 11% mobile phase B, then from 10 to 40 min a linear gradient from 11 to 28% B. The flow-rate was maintained at 0.80 ml/min and the column temperature was 50°C. The effluent was monitored at 240 nm. Mobile phase A consisted of methanol–water (30:70, v/v); mobile phase B consisted of methanol–acetonitrile (90:10, v/v). The mobile phases were adjusted to pH 4.5 by the addition of acetic acid.

The quantitation of the testosterone metabolites was performed using the peak heights measured at 240 nm and was corrected for the internal standard 11β-OH-T. The metabolites for which a standard was available were further calculated to concentration units using a standard curve. For the metabolites for which no standard was available, an average absorption efficient of 0.470 (for a solution of 1 μg/ml) was used.

RESULTS

In the first experiment, several endogenous steroids were tested for their ability to serve as substrates for monitoring the isoenzyme pattern of cytochrome P-450. The steroids were incubated with microsomes from control rats, and after extraction, the product formation was monitored with reversed-phase HPLC. The parameters monitored were the extent of substrate consumption of the particular steroid used, the number of metabolites observed in the chromatogram and the relative amounts of the observed metabolites. The following steroids were tested: 17β-testosterone,

3,17-androstenedione, progesterone, 17β-estradiol and cortisol. For 17β-estradiol and cortisol only a few small peaks were observed in the chromatogram. For testosterone, progesterone and androstenedione a number of metabolites were observed, some in relatively high amounts. A total of 28% of the testosterone was metabolised, resulting in four peaks in relatively high amounts (2.7–7.2% relative to the starting amount of testosterone). In addition, eight smaller peaks were observed in the order of 0.25 and 0.6%. Only 7% of the progesterone was degraded, which resulted in four large peaks, ranging from 0.2 to 2.9%, and three smaller peaks. A total of 23% of the androstenedione was metabolised, which resulted in two large peaks of 4.8 and 7.3%, and six smaller peaks in the order of 0.15–0.4% of the starting amount of androstenedione. Based on this information from the chromatograms, 17β-testosterone was selected for further studies.

The assay with 17β-testosterone was developed into a routine assay using microsomes from male rats treated with dexamethasone; these microsomal preparations produced the largest number of peaks in the chromatogram. It is shown in Fig. 1 that, in addition to the internal standard 11β-OH-T, eleven peaks can be observed which are not found in the blank experiment. Three or four very small peaks can also be observed. By comparing these with various hydroxylated testosterone standards which were obtained commercially, seven peaks could be identified: 6β-OH-T at 9.45 min, 19-OH-T at 10.35 min, 7α-OH-T at 11.28 min, 16α-OH-T at 15.38 min, 16β-OH-T at 17.63 min, 11α-OH-T at 19.18 min and androstenedione at 33.73 min. The peaks with retention times of 23.26 and 25.03 min could be assigned to 2α-OH-T and 2β-OH-T, respectively, by comparison with literature data [8,10] in combination with

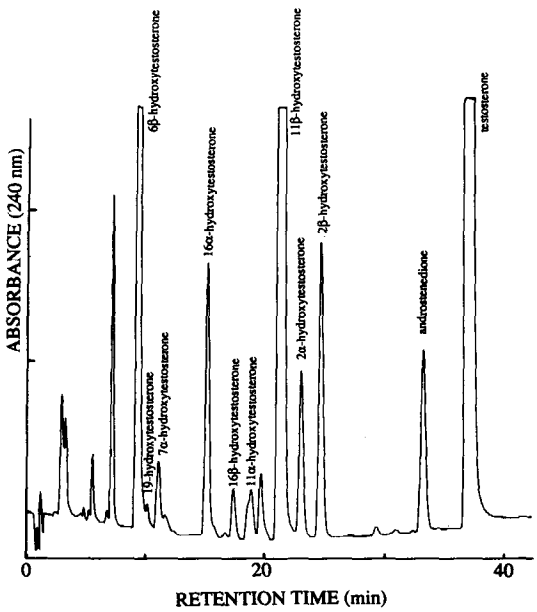

Fig. 1. HPLC pattern of the incubation mixture of 17β-testosterone with microsomes of a rat treated with dexamethasone. The identified hydroxylated testosterone metabolites are indicated in the chromatogram.

the results obtained with microsomes from control rats. The 2α-OH-T peak is large in control microsomes and decreases in intensity on induction by dexamethasone, phenobarbital and β-naphthoflavone. The 2β-OH-T peak shows a low intensity in the control microsomes, whereas an increase is observed on induction by dexamethasone and phenobarbital. The relative retention times are in agreement with reported data. The remaining major peaks that are still to be identified have retention times of 5.71, 7.31 and 19.98 min, there are small unidentified peaks at 11.86 and 29.76 min.

The quantitation of the metabolites was performed by comparison of the HPLC peak heights with those of available standards. Hydroxylated testosterone metabolites for which no standard was available (2α- and 2β-OH-T) were quantitated using an average absorption coefficient of 0.470, which was calculated from seven hydroxylated testosterone standards. The absorption coefficients and maximum wavelengths are summarised in Table I. For all standards the working range was checked and showed a perfectly linear behaviour between 50 and 800 ng of the steroid injected, with correlation coefficients in the range 0.995–0.999. The limit of detection for the hydroxylated steroids was about 3 ng per injection with a signal-to-noise ratio of 3. This value was extrapolated from a 50-ng amount.

TABLE I

MAXIMUM WAVELENGTH AND ABSORPTION COEFFICIENTS (1 μg/ml SOLUTIONS) OF 17β-TESTOSTERONE AND METABOLITES

Steroid[a]	Absorption coefficient at 240 nm	Maximum wavelength (nm)	Maximum absorption coefficient
T	0.565	247.8	0.647
6β-OH-T	0.499	241.2	0.505
7α-OH-T	0.555	247.0	0.623
11α-OH-T	0.485	247.4	0.542
11β-OH-T	0.350	247.2	0.393
16α-OH-T	0.388	248.6	0.462
16β-OH-T	0.478	247.5	0.540
19-OH-T	0.388	248.6	0.462
AD	0.553	246.1	0.600

[a] T = 17β-testosterone; AD = 3,17-androstenedione.

The pH dependence of the hydroxylation of testosterone was investigated for some metabolites using microsomes from a control male rat. Table II gives the results for pH values 6.9, 7.4, 7.9 and 8.4. It appears that the various stereo-specific hydroxylation reactions show different pH behaviours. For all further experiments a pH of 7.4 was chosen as a compromise.

The kinetics of the hydroxylation reaction was investigated by monitoring the relative peak heights as a function of time. The results of this experiment are shown in Fig. 2 for the hydroxylation of testosterone by microsomes from male rats which have been treated with dexamethasone. All the curves showed a linearity from 0 to 15 min. An incubation time of 15 min was therefore chosen for all further experiments.

TABLE II

pH DEPENDENCE OF THE FORMATION OF METABOLITES OF 17β-TESTOSTERONE

Determined with microsomes from a control male rat. Data are expressed in arbitrary units as peak heights, corrected for the internal standard 17β-OH-T.

Metabolite	Peak height			
	6.9	7.4	7.9	8.4
2α-OH-T	409	469	371	341
2β-OH-T	13.2	26.8	31.6	32.7
6β-OH-T	202	526	681	746
7α-OH-T	109	127	89	77
16α-OH-T	531	672	581	539
16β-OH-T	20.3	31.2	27.4	27.1
AD[a]	585	666	618	597

[a] 3,17-Androstenedione.

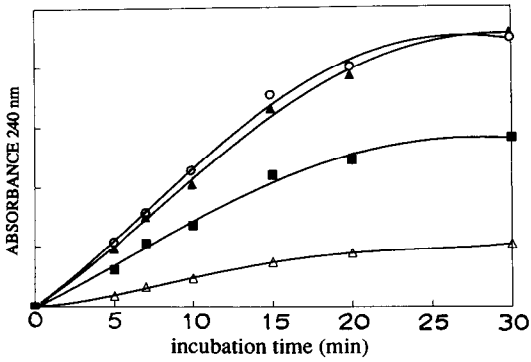

Fig. 2. Kinetic behaviour of the formation of four metabolites of 17β-testosterone as a function of incubation time as determined with microsomes from a male rat treated with dexamethasone. (■) 2α-OH-T; (△) 7α-OH-T; (▲) 6β-OH-T; and (○) 16β-OH-T.

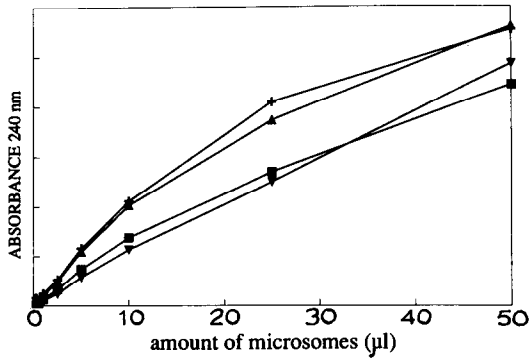

Fig. 3. Linearity of the formation of four metabolites as a function of the volume of rat liver microsomes in the range 0.5–50 µl. (+) Androstenedione; (▲) 16α-OH-T; (■) 2α-OH-T; and (▼) 6β-OH-T.

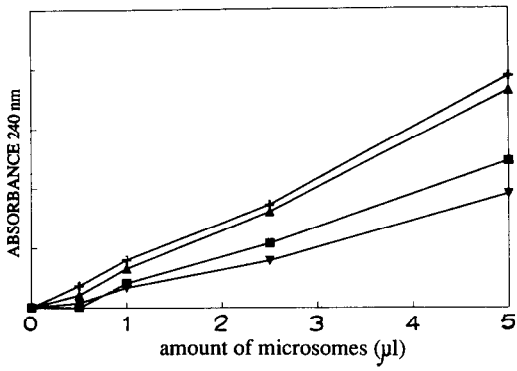

Fig. 4. Linearity and determination of the limit of detection of the formation of four metabolites as a function of the volume of rat liver microsomes in the range 0.5–5 μl. Symbols as in Fig. 3.

To determine the linearity on dilution and the limits of detection with respect to the amount of microsomes, dilution experiments were performed with microsomes from both control male rats and male rats treated with dexamethasone. An approximate linear behaviour was obtained, with 50 μl of microsomes as an upper and 5–0.5 μl of microsomes as a lower detection limit, depending on the peak height (Fig. 3). High-intensity peaks could be observed down to a 200-fold dilution of the microsomal preparations in the buffer, resulting in an absolute amount of 0.5 μl of microsomes (Fig. 4), whereas peaks of low intensity could be detected with 5 μl of microsomes. The criterion used for peak detection was the indication of peaks by the integrator. In practice the limits of detection can be decreased further by changing the integration parameters. An example of such a dilution experiment is shown in Fig. 4 for four HPLC peaks after the incubation of testosterone with microsomes from a control rat.

DISCUSSION

The occurrence of the various cytochrome P-450 isoenzymes can be monitored by product formation in a real enzymatic assay in addition to other analytical detection techniques such as electrophoresis followed by Western blotting with immunochemical detection. Steroids were shown to be good substrates for the cytochrome P-450 system. 17β-Testosterone and progesterone are good substrates which can be hydroxylated at various stereochemically different sites. HPLC proved to be very suitable to detect and quantitate the various metabolites. The results of this study indicate that twelve peaks can be observed in a simple reversed-phase gradient system. By comparison with standards, eight of these peaks could be assigned to several hydroxylated isomers of testosterone and to androstenedione. Two more peaks could be identified based on comparison with data from the literature, combined with experiments with microsomes from control rats. A few peaks still remain to be identified.

In addition to a number of cytochrome P-450 isoenzymes, the rat liver microsomes also contain other enzymes which can act on endogenous steroids, such as

5α-reductase. This enzyme renders the 3-keto-4-ene moiety into a saturated A-ring structure. The resulting 3-keto- or 3-hydroxyandrostanes cannot be detected by HPLC because of the absence of a sensitive ultraviolet-absorbing group. A predominant activity of 5α-reductase is present, especially in microsomes from female rats [16]. As a result, the assay described here cannot be used for the assessment of the cytochrome P-450 isoenzymes in female rats. Incubation of testosterone with microsomes from female rats results in complete consumption of testosterone and the formation of androstanes, which results in an "empty" chromatogram. Although the activity of 5α-reductase is much lower in male rats, the influence of this enzyme cannot be completely ignored. Possible effects on the quantitative determination of different cytochrome P-450 isoenzymes can be expected. Therefore the addition of 5α-reductase inhibitor is probably a prerequisite for good quantitation of the real enzymatic activity.

To assign the formation of the different hydroxylated testosterone metabolites to the presence of certain specific isoenzyme forms of cytochrome P-450, a large amount of data from the literature has been combined. Isoenzyme P-450a (new nomenclature: IIA1 [12]) is specifically involved in the formation of 7α-OH-T [15,17–21]. Isoenzyme P-450b (IIB1) metabolises testosterone into the metabolites 16α-OH-T, 16β-OH-T and androstenedione. The formation of 16β-OH-T is specific for this isoenzyme. Isoenzyme P-450c (IA1) hydroxylates testosterone on the 6β- and 2β-position [15,17,20]. As this isoenzyme has a very low hydroxylase activity towards testosterone, the contribution to the metabolic pattern will be low. For isoenzyme P-450d (IA2) the same, but lower, activities were found as for isoenzyme c. As a result, isoenzymes c and d, which are important in the assessment of exposure to polychlorinated hydrocarbons, cannot be detected with the testosterone assay. Isoenzyme P-450e (IIB2) shows the same metabolic pattern as isoenzyme b, but has only about 10% of its activity [20]. With isoenzyme P-450g (IIC13) the metabolites 6β-OH-T, 15α-OH-T and 16α-OH-T are formed, but in low amounts [17]. Isoenzyme P-450h (IIC11) catalyses the specific formation of 2α-OH-T and also the less specific metabolite 16α-OH-T [15,17–19]. Isoenzyme P-450p (IIIA1) forms four hydroxylated metabolites of testosterone, 2β-OH-, 6β-OH-, 15β-OH- and 18-OH-T [15,19]. As this isoenzyme is responsible for more than 85% of the 6β-hydroxylase activity, the occurrence of this metabolite can be ascribed to isoenzyme P-450p.

In conclusion, it can be stated that with the testosterone hydroxylase assay the following cytochrome P-450 isoenzymes can be quantitated based on their enzymatic activity: isoenzymes P-450 a (7α-OH-T), b (16β-OH-T), h (2α-OH-T) and p (6β-OH-T). For this very sensitive assay only a limited amount (0.5 μl) of microsomes is required. Further studies, including more statistical evaluations and practical applications, will be performed as soon as inhibitors of 5α-reductase become available.

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