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TESTOSTERONE METABOLITE PROFILES REVEAL DIFFERENCES IN THE SPECTRUM OF CYTOCHROME P-450 ISOZYMES INDUCED BY PHENOBARBITONE, 2-ACETYLAMINOFLUORENE AND 3-METHYLCHOLANTHRENE IN THE CHICK EMBRYO LIVER

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Abstract—The regiospecificity and stereoselectivity of testosterone hydroxylation by hepatic microsomes prepared from control, PB, 3MC and 2AAF treated chick embryos has been analysed. Microsomes prepared from control animals hydroxylate testosterone at the 16α and 6β positions exclusively: 3MC treatment only causes comparatively minor alterations in the rates of these conversions. PB and 2AAF treatment induced 16β-hydroxylation, whilst only 2AAF caused a substantial induction of 6β hydroxylation. This data suggests that in the chick, 2AAF not only induces P-450 subforms which are also induced by PB but additional subforms which are not markedly induced by either PB or 3MC.

We have recently demonstrated that administration of the potent hepatocarcinogen 2AAF‡ to chick embryos produces a three-fold induction in hepatic cytochrome P-450 content [1]. As cytochrome P-450 subforms are involved in both the activation and detoxification of 2-acetylaminofluorene (2AAF) we have proposed that the chick embryo may be a useful model in which to study the relationship between the hepatic cytochrome P-450 population and 2AAF toxicity [2]. To catalytically characterise the cytochrome P-450 subforms induced by 2AAF in chick embryo liver we compared the effects of phenobarbitone (PB), 3-methylcholanthrene (3MC) and 2AAF pretreatment on microsomal *N*-demethylation, *O*-dealkylation and aromatic hydroxylation; we also examined the regiospecificity of microsomal 2AAF hydroxylation itself after each of these treatments [3]. This study suggested that 2AAF induced cytochrome P-450 subforms which are individually induced by PB or 3MC treatment; thus the induction effect of 2AAF resembled that of arocolar 1254 [4]. Subsequently Astrom and De Pierre, using antibodies to cytochrome P-450b, P-450c, P-450d and P-450PCN (the major subforms induced by combined PB and 3MC treatment of rats) reported that 2AAF treatment of rats induces each of these cytochrome P-450 subforms [5]. Thus in both the rat and chick embryo 2AAF acts as a mixed PB/3MC type inducer, though it should be noted that 2AAF is a more potent cytochrome P-450 inducer in the chick embryo than in the adult rat [1].

Several recent reports have shown that purified cytochrome P-450 subforms are capable of hydroxylating testosterone with a high degree of regiospecificity and stereoselectivity in reconstituted systems [6, 7]. This has suggested that the analysis of hepatic microsomal testosterone hydroxylation specificity may provide a rapid and high-resolution method of assessing the spectrum of cytochrome P-450 subforms present. Our previous studies have shown that chick embryo hepatic microsomes possess a defined and limited capacity to hydroxylate testosterone at the 16α and 6β-positions [8]. In this report we examine the effects of PB, 2AAF and 3MC treatment on the extent and direction of testosterone hydroxylation and demonstrate that 2AAF induces a distinctive spectrum of catalytic activities.

MATERIALS AND METHODS

Fertilised hens eggs of the Ross 1 strain were obtained from W. C. Blacklocks Ltd., Lydd, Kent. Sources of specialist materials were as follows: Amersham International, [4-^{14}C]testosterone (sp. act. 50 mCi/mmol); Eastman–Kodak, silica gel TLC plates with fluorescent indicator; Fisons, HPLC grade solvents; Waters Associates, Sep-Pak C-18 cartridges. We thank Professor D. N. Kirk (MRC Steroid Reference Collection, Department of Chemistry, Queen Mary College, London) for providing authentic samples of hydroxylated testosterones. All other reagents were from the Sigma Chemical Company and of the highest purity available.

Treatment of animals and microsome preparation.

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‡ Abbreviations: PB, phenobarbitone; 3MC, 3-methylcholanthrene; 2AAF, 2-acetylaminofluorene; 16α-OHT, 16α-hydroxytestosterone; 6β-OHT, 6β-hydroxytestosterone; 16β-OHT, 16β-hydroxytestosterone; TMS, trimethylsilylchloride; DMSO, dimethylsulphoxide.

Seventeen days after the eggs were placed in the incubator, inducing agents were administered in 0.2 ml of DMSO into the fluid surrounding the embryo at the following doses: PB, 10 mg; 2AAF, 8 mg; 3MC, 2 mg. Controls received vehicle alone. Twenty-four hours later the livers were removed and microsomes prepared as described previously [1].

Testosterone hydroxylation assay. The incubation mix for testosterone conversion assays provided conditions under which formation of all the hydroxylated metabolites was linear with respect to both assay time and protein content and consisted of 20 mM sodium phosphate pH 7.4; 5 mM $MgCl_2$, 5 μM $MnSO_4$, 5 mM isocitric acid, 0.5 mM NADP, 0.5 units isocitrate dehydrogenase and 1 mg microsomal protein in a final volume of 1 ml at 37°. Testosterone was added to incubations in 20 μl of methanol to a final concentration of 0.1 mM; when metabolites were to be analysed by TLC up to 4×10^5 dpm of [4-^{14}C]testosterone was added to the assay. After 30 min the reaction was stopped by the addition of 1 ml of cold methanol and the precipitated protein removed by centrifugation (2000 *g* for 15 min at 4°). The supernatant was recovered and diluted with water so that the final methanol content was 25% (v/v). This was applied to a Sep-Pak C-18 reverse phase cartridge which had been primed with 2 ml of methanol and 5 ml of water. After sample application the cartridge was washed with 2 ml of 25% methanol (v/v) and absorbed steroids eluted with methanol.

HPLC analysis of testosterone metabolites. HPLC analysis was performed using a Varian 5000 system fitted with a 25 cm Whatman Partisil 5-ODS3 column and a 5 cm Co. Pell ODS guard column. 0.1 ml of the metabolite sample in methanol was injected onto the column; elution of hydroxylated testosterones was achieved with a mobile phase of 43% methanol–52% water–5% acetonitrile at a flow rate of 1.6 ml/min; after 30 min the mobile phase was changed to 100% methanol to elute testosterone and androstenedione. Steroids were detected by absorbance at 240 nm using a Varian UV-5 monitor; retention times and peak areas were determined using a Varian CDS 111L computing integrator.

TLC analysis of testosterone metabolites. Methanolic eluates from Sep-Pak cartridges were dried under vacuum and resuspended in dichloromethane. Samples were applied as 2 cm lines to silica gel G TLC plates (thickness 0.1 mm), which had been preactivated at 80° for 30 min. Testosterone metabolites were resolved by developing the plate twice in a solvent system of dichloromethane–acetone 70:30 (v/v). Radiolabelled metabolites were detected by autoradiography using Kodak X-OMAT RP5 film and were initially identified by their co-migration with standards (visualised by fluorescence quenching) which were applied to the same TLC plate.

Mass spectroscopy. TMS derivatives of steroids (1–10 μg) recovered from TLC plates were prepared using hexamethyldisilazane and trimethylsilylchloride in pyridine [10]. Samples were introduced into the mass spectrometer via the probe which was heated from 25° to 300° over a period of 8 min; volatilised matter was ionised by electron impact at 70 eV.

Other assays. Microsomal cytochrome P-450 and protein content were determined as previously described [1].

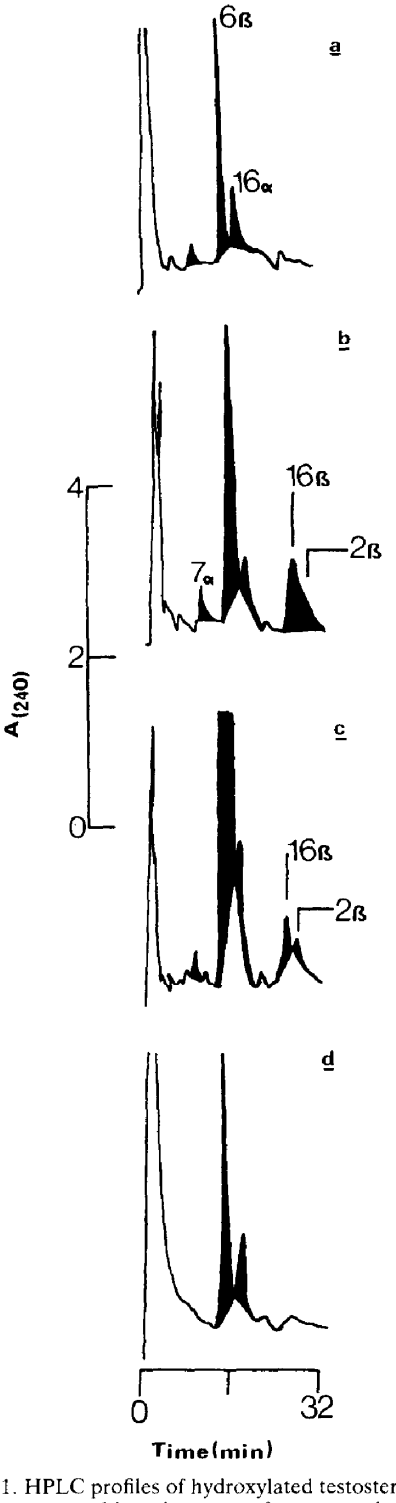

Fig. 1. HPLC profiles of hydroxylated testosterone metabolites generated by microsomes from control and induced animals. Testosterone metabolites generated by microsomes prepared from control (a), PB (b), 2AAF (c) or 3MC (d) induced animals were resolved using reverse phase chromatography.

Table 1. Retention times and mobilities of hydroxylated testosterones

Position of hydroxylation	TLC mobility (^{R}Testosterone)	HPLC retention time (min)
2α	0.62	25.7
2β	0.55	27.9
6α	0.60	9.8
6β	0.73	15.4
7α	0.41	12.0
11α	0.24	19.4
11β	0.49	25.8
14α	0.47	15.2
15α	0.30	n.d.
16α	0.20	17.1
16β	0.65	26.8
18	0.69	27.5
19	0.52	14.3

n.d.—not done.

RESULTS AND DISCUSSION

In this study we have examined the capacity of hepatic microsomes, prepared from chick embryos, to perform testosterone hydroxylation. The assay system we use has been described elsewhere [8]. Under the conditions we used, product formation was linear for up to 60 min and protein concentrations up to 1 mg/ml. There were no differences in the rate of metabolite formation when the substrate concentration was increased five-fold (data not shown).

As part of this investigation we have developed HPLC and TLC systems which can resolve up to 13 monohydroxylated testosterones. Retention times (RT) of TLC metabolites relative to testosterone are given in Table 1. Given that neither technique was able to resolve all of the (authentic) standard metabolites we regard these as complementary approaches to the resolution of metabolites from experimental systems. Thus although two of the five metabolites (16β-OHT and 2β-OHT) generated by the assay mixture were not completely resolved by HPLC (Table 1 and Fig. 1) all of the metabolites under study were resolved by TLC (Table 1). The value of HPLC lies in its ability to provide a rapid ($t < 40$ min) metabolite fingerprint.

The initial identification of metabolites was based on their co-elution or co-migration with authentic reference compounds. Positive identification was by mass spectroscopic analysis, however; a more detailed discussion of the procedure as applied to TLC is given by Darby *et al.* [8]. Central to the analysis was the extraction and recovery of testosterone and its hydroxylated derivatives using Sep-Pak cartridges. This was a key step since it removed the less-polar lipid material which is known to impair the efficiency of HPLC [9]. Recovery of metabolites and substrate was routinely greater than 85%, as determined from the total counts eluted from the cartridge and TLC strips. To ensure that the extraction and concentration steps did not alter the distribution of metabolites we standardised the system using authentic standards of the known major hydroxy testosterone products of chick hepatic microsomes (Table 2). There was no alteration in the ratio of 6α-OHT, 6β-OHT, or 16α-OHT with respect to 7α-OHT (a minor metabolite) in a reference mixture processed under the standard assay conditions. We concluded that the extraction and analysis procedures were both specific and highly reproducible.

Representative HPLC profiles of metabolites formed by hepatic microsomes from control and xenobiotic treated chick embryos are shown in Fig. 1. The major metabolites produced by control microsomes were 6β-OHT and 16α-OHT (Fig. 1a), a finding consistent with our previous study [8]. No sex differences were found in the rates of formation of any metabolite by microsomes (from control or induced animals); consequently we used microsomes from pooled livers for further study. Treatment of animals with PB, 3MC or 2AAF had a marked effect on hepatic microsome testosterone hydroxylating capacity (Figs 1b–d). PB treatment produced a marked increase in the production of 6β-OHT, 16β-OHT, 2β-OHT and 7α-OHT. A similar response was noted on treatment of animals with 2AAF except that 6β-OHT production was stimulated to a greater extent; compare Fig. 1b with Fig. 1c. Treatment with 3MC did not appear to alter the metabolite profile; compare Fig. 1a with Fig. 1d. A measure of these changes was made from TLC analysis (Table 3). None of the treatments caused a significant alteration ($P > 0.05$) in the rate of 16α-OHT formation. 6β-OHT formation was significantly increased after 3MC (1.7-fold; $P < 0.05$), PB (2.1-fold; $P < 0.05$) or 2AAF (8.7-fold; $P < 0.01$) treatment. Metabolite identifications were confirmed at this stage by GC-mass spectroscopy. The TMS derivative of the putative 6β-OHT produced major mass fragments of *m/e* 448 (M^+), 433, 329 and 129 consistent with its assigned structure [10].

Table 2. Recovery of hydroxylated testosterones from Sep-Pak cartridges

Testosterone metabolite	Ratio with respect to 7α-hydroxytestosterone	
	Standard mixture	Sep-Pak eluate
6α	0.52–0.55	0.50–0.55
6β	0.45–0.50	0.44–0.51
16β	0.47–0.51	0.50–0.53

A mixture of hydroxylated testosterones was added to the standard assay mix and processed as described in the text. Metabolites were resolved by HPLC and quantified by integrating the area under the peaks. Values indicate the range of area ratios obtained from 3–5 experiments.

Table 3. The effects of xenobiotics treatment on the production of 6β-hydroxy, 16α-hydroxy, and 16β-hydroxytestosterone by microsomes

Treatment	Testosterone metabolite production (units mg/protein/min) 16α-OHT	6β-OHT	16β-OHT
Control	8.0 ± 1.4	25.0 ± 4.2	b.d.
PB	9.6 ± 1.6	54.1 ± 7.8*	25.9 ± 1.1
2AAF	8.8 ± 1.6	212.0 ± 30.0**	12.7 ± 0.8
3MC	9.8 ± 1.6	42.6 ± 4.2*	b.d.

Each value is the mean ± S.D. of assays on at least four microsomal preparations. Microsomal cytochrome P-450 concentrations were as follows: control 177 ± 12 pmol/mg; PB, 908 ± 122 pmol/mg; 3MC, 378 ± 29 pmol/mg; 2AAF 605 ± 73 pmol/mg.
b.d.—below detectable.
* $P < 0.05$, ** $P < 0.01$.

Formation of 16β-OHT by microsomes prepared from control and 3MC treated animals was undetectable. Microsomes from PB or 2AAF treated animals showed marked production of this metabolite. Again the mass fragmentation pattern [*m/e* 448 (M^+), 358, 316, 268 and 191] was consistent with its assigned structure [6]. The similarities between 2AAF and PB in terms of the two minor metabolites which were produced, 2β-OHT and 7α-OHT, are tentative at this stage given that we could not recover sufficient of these compounds for mass spectroscopic analysis.

The data we present in this report is of interest in two main areas. Firstly it illustrates the complementary nature of HPLC and TLC in the analysis of testosterone metabolites and secondly the usefulness of testosterone metabolite fingerprints in comparing induction responses.

With regard to the latter we have shown that treatment of chick embryos with 2AAF or PB causes marked alterations in the rate and direction of testosterone hydroxylation by hepatic microsomes; in contrast 3MC treatment causes no alteration in the direction and only a small increase in the overall rate. These findings suggest similarities in the properties of rat and chick embryo hepatic cytochrome P-450 subforms. The rat 3MC inducible P-450c and P-450d subforms are poor catalysts of testosterone hydroxylation and 3MC treatment of rats causes little alteration in the rate or specificity of conversion [7]. However, in both the rat and chick embryo PB treatment induces microsomal testosterone 16β-hydroxylation; in the rat this conversion is performed by the PB inducible P-450b and P-450e subforms [7]. It is interesting to speculate that if a similar spectrum of isozymes were induced in both species it could be a reflection of the fact that the gene families involved have been highly conserved.

The observation that 2AAF treatment of chick embryos also induces 16β-hydroxylation supports our previous suggestion that both PB and 2AAF may induce some of the same cytochrome P-450 subforms. 2AAF was a more specific inducer of testosterone 6β-hydroxylation than either PB or 3MC. This may reflect the induction of a chick embryo hepatic cytochrome P-450 subform by 2AAF which is not markedly induced by PB or 3MC. Indeed we previously reported that 2AAF causes a unique alteration in the electrophoretic profile of microsomal proteins which may result from the induction of a cytochrome P-450 apoprotein distinct from any induced by PB or 3MC [1].

In conclusion, the data presented here and in previous studies [1–3] suggests that 2AAF induces chick embryo hepatic cytochrome P-450 subforms which are also induced by PB and 3MC and at least one cytochrome P-450 subform which is not markedly induced by either of these xenobiotics. In support of this view it has recently been demonstrated that 2AAF acts as a mixed PB-3MC type inducer in the rat [5]. This study also illustrates the usefulness of testosterone metabolism in the rapid fingerprint analysis of the spectrum of cytochrome P-450 in microsomes. The use of both HPLC and TLC provides speed, resolution and quantitation.

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