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USE OF A DIRECT HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR MULTIPLE TESTOSTERONE HYDROXYLATIONS IN STUDIES OF MICROSOMAL MONOOXYGENASE ACTIVITIES

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Abstract—A high performance liquid chromatography method is described for separation of the products of testosterone monooxygenation. The method involves direct injection onto a reverse phase octadecylsilane column of the supernatant from incubations containing as little as 25 μg microsomal protein. Isocratic elution with formate buffer/acetonitrile and detection at 254 nm permits the separation and simultaneous quantitation of multiple discrete testosterone-derived peaks on the chromatogram. The production of the eight major oxygenated testosterone metabolites in hamster liver microsomes was determined. This profile was altered uniquely after pretreatment with either phenobarbitone, β-naphthoflavone, rifampicin or pregnenolone 16α-carbonitrile. These changes reflected analogous dissimilar effects of the enzyme inducers on the metabolism of the exogenous cytochrome P-450 substrates aldrin, acetanilide, benzo[α]pyrene and ethylmorphine. Therefore, the testosterone assay provides a sensitive and effective method for separating and quantitating testosterone monooxygenation products and may offer a single alternative to the use of multiple exogenous substrates for categorizing and defining the metabolic activity of cytochromes P-450.

Cytochrome P-450, the terminal electron acceptor for microsomal monooxygenases, exists in multiple forms each of which has discrete but overlapping substrate specificities [1]. Comprehensive measurements of the metabolic expression of this heterogeneity in liver microsomes have predominantly employed model xenobiotic cytochrome P-450 substrates such as benzo[α]pyrene, biphenyl and ethylmorphine although endogenous substrates have been used to complement the process [2–4]. Use of exogenous substrates presents few problems in studies with experimental animals, but their inherent toxicity limits their usefulness *in vivo*. In situations where tissue supplies are limited, e.g. in man or in long-term tests with animals, it may also be impossible to perform as many *in vitro* assays of exogenous substrate oxygenations as would be required for a comprehensive evaluation of hepatic monooxygenase activity or the effects of administered drugs or environmental contaminants.

Specific microsomal testosterone hydroxylations have been widely used as *in vitro* monitors of enzyme induction [3, 5–8]. The 2β-, 6β-, 7α- and 16α-hydroxytestosterones have been considered the major products of the cytochrome P-450 catalysed oxygenation of testosterone [9]. However, other hydroxytestosterones are produced and these may assume quantitatively greater importance after enzyme induction [10–13]. In the past we have employed a radiometric assay for testosterone hydroxylations in which metabolite identification is achieved by chromatography with known standards on a thin-layer silica gel coated plate [7]. Since the amounts of metabolic products are small, direct visualization is usually impossible and this can introduce errors when bands are scraped off the plate, particularly if development is not uniform and unexpected metabolites are produced. Van der Hoeven [11], Newton and colleagues [14] and Wood *et al.* [13] have recently reported the use of high pressure liquid chromatography methods for the detection of several hydroxytestosterones but these assays still involve solvent extraction which in our experience caused a loss of reaction linearity at lower protein concentrations. We have overcome this problem by developing a high performance liquid chromatography method in which the products of microsomal testosterone oxygenation can be injected directly onto a reverse phase octadecylsilane column.

In this report we describe the direct high performance liquid chromatography assay and its application to a study of the differential effects of phenobarbitone, β-naphthoflavone, pregnenolone 16α-carbonitrile and rifampicin on the monooxygenation of testosterone in hamster liver microsomes. To evaluate the usefulness of the method we have compared results obtained with testosterone and those using the four exogenous model substrates ethylmorphine, aldrin, benzo[α]pyrene and acetanilide.

MATERIALS AND METHODS

Chemicals. Androstenedione (4-androstene-3,17-dione), bovine serum albumin (fraction V), cytochrome *c*, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, $NADP^+$, β-naphthoflavone (5,6-benzoflavone) and paracetamol were obtained from

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Sigma, Poole, Dorset, England; acetanilide, formaldehyde, *n*-hexane (for pesticide residue analysis), *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid (Hepes), phenobarbitone sodium, semicarbazide and Tris(hydroxymethyl)aminomethane from Hopkin and Williams, Romford, Essex, England; acetonitrile (HPLC grade S) from Rathburn Chemicals Limited, Walkerburn, Peeblesshire, Scotland; benzo[α]pyrene from Aldrich Chemical Company Ltd., Gillingham, Dorset, England; Endrin (> 99% 1, 2, 3, 4, 10, 10-hexachloro-6,7-epoxy-1, 4, 4a, 5, 6, 7, 8, 8a-octahydro - 1,4 - endo - 5,8 - endo - dimethanonaphthalene) and 1.5% OV 17 + 1.95% OV 202 silicones on Chromsorb WHP 80 from Chrompack UK Ltd., London, England; carbon monoxide from Cambrian Chemicals, Croydon, England and testosterone (4-androsten-17β-ol-3-one), 1-dehydrotestosterone and 6β-, 7α- and 16β-hydroxytestosterones from Steraloids Ltd., Croydon, England. Ethylmorphine hydrochloride was donated by May and Baker, Dagenham, Essex, England; rifampicin by Ciba Laboratories, Horsham, West Sussex, England; pregnenolone 16α-carbonitrile (16α-cyano-5-pregnen-3β-ol-20-one) by G. D. Searle and Co., Chicago, IL, U.S.A.; Aldrin (>99% 1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4-endo-5,8-exo-dimethanonaphthalene) and dieldrin (> 99% 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-endo-5,8-exo-dimethanonaphthalene) by Shell Research Ltd., Sittingbourne, Kent, England; 2α-, 2β-, 6α-, 15α-, and 16α-hydroxytestosterones by courtesy of Professor D. N. Kirk, The Medical Research Council Steroid Reference Collection, Westfield College, London, England.

Animals and pretreatment regimens. Male Syrian Golden hamsters aged 9 weeks and weighing ~ 100 g were obtained from Wrights of Essex, Chelmsford, Essex, England. They were housed four per cage on dried sugar beet pulp bedding (British Sugar Corporation, Peterborough, Cambridgeshire, England) and fed on C.R.M. Diet (Rank-Hovis-McDougall, Poole, Dorset, England) and tap water *ad libitum* for two weeks before treatment. All inducing agents were given by intraperitoneal injection in the appropriate vehicle (5 ml/kg body wt) using the following regimens: β-naphthoflavone (80 mg/kg in corn oil): one dose 48 hr before killing; phenobarbitone (80 mg/kg in saline), pregnenolone 16α-carbonitrile (40 mg/kg in corn oil) or rifampicin (50 mg/kg in HCl, pH 3): one dose on each of three consecutive days with the final injection 24 hr before killing. Control animals were injected on three consecutive days with saline alone since we have detected no significant difference between the effects of pretreatment with corn oil or saline on our assays.

Tissue preparation. Hamsters were killed by cervical dislocation and washed hepatic microsomal pellets were prepared as described previously [15]. After resuspension of the pellet in 1.5 vol 1.15% KCl containing 20% glycerol, 1 mM EDTA and 10 mM Hepes buffer, pH 7.6, protein concentrations were determined by the method of Lowry *et al.* [16]. The microsomal suspensions were then frozen and stored in liquid nitrogen, conditions under which there were no significant changes in enzyme activities [17].

Biochemical determinations. Cytochrome P-450 concentrations were measured in microsomal suspensions containing 2 mg microsomal protein/ml 0.1 M Hepes buffer, pH 7.6 + 20% glycerol by the method of Omura and Sato [18] using a Cecil Instruments (Cambridge, England) CE 5095 dual beam spectrophotometer. NADPH-Cytochrome *c* reductase activities were determined at 37° using the same spectrophotometer to measure the reduction of cytochrome *c* at 550 nm as described by Peters and Fouts [19]. The incubation was performed in 0.1 M Tris buffer, pH 8.0 but otherwise used the assay conditions described previously [7]. Minor modifications of the methods of Bend *et al.* [20], Shimazu [21] and Wattenberg *et al.* [22] were used in the assays for ethylmorphine *N*-demethylase, acetanilide 4-hydroxylase and benzo[α]pyrene hydroxylase, respectively. The components of the incubation mixtures and modifications used have been described previously [7]. The only additional change in conditions was that 2 M HCl/20% trichloroacetic acid was used to stop the acetanilide hydroxylase assay since this mixture gave optimal results as both a protein precipitant and hydrolytic agent.

Aldrin epoxidation. The microsomal epoxidation of aldrin was assayed using modifications of the method of Wolff *et al.* [23]. Incubations were performed in screw-topped glass tubes and contained 100 μmole Hepes buffer, pH 7.6, 2.5 μmole glucose 6-phosphate, 5 μmole $MgCl_2$, 0.5 μmole $NADP^+$, 0.5 units glucose 6-phosphate dehydrogenase, microsomal protein and water to a vol of 980 μl. After 5 min preincubation at 37° the reaction was started by the addition of 20 μl methanol containing 200 nmole aldrin and allowed to proceed for 5 min before adding 5 ml hexane, shaking vigorously and plunging the tubes into ice. The appropriate amount of endrin internal standard was then added and the tubes were capped and shaken on a rotary shaker for 30 min. The hexane layer was separated by centrifugation (1500 *g*, 10 min, 4°) and immediately aspirated into fresh tubes which were sealed and stored at 4° before analysis. Under these conditions the production of dieldrin was linear both with respect to protein concentration and time and the extraction of both dieldrin and endrin from the incubation mixture was complete. An aliquot of the hexane extract containing 400 pg endrin was injected onto a 2.5 m × 6.4 mm (o.d.) × 2 mm (i.d.) glass column packed with 1.5% silicone OV 17 + 1.95% silicone OV 202 on chromsorb WHP 80 mesh and mounted in a Pye 104 gas chromatograph (Pye, Unicam, Cambridge, England). Oven temperature was 210°, detector temperature 270°. Nitrogen carrier gas was used at a flow rate of 20 ml/min through the column and this was supplemented with a purge of an additional 20 ml/min through the ^{63}Ni electron capture detector. Using these conditions, aldrin, dieldrin and endrin had retention times of 3.6, 8.6 and 10.4 min, respectively. Quantitation of dieldrin was achieved by determining the ratio of the areas (height × width at half-height) of the dieldrin to endrin peaks and by reading from a linear standard curve prepared using 400 pg endrin and 20–200 pg dieldrin.

Testosterone hydroxylation. The incubation mixture for testosterone hydroxylations provided condi-

tions for assay in which linearity with respect to both protein and time was achieved and comprised 200 μmole Hepes buffer, pH 7.6, 5 μmole glucose 6-phosphate, 10 μmole $MgCl_2$, 0.1 μmole $NADP^+$, 1 unit glucose 6-phosphate dehydrogenase, 100 μg microsomal protein and water to 1.98 ml. After 5 min preincubation at 37° the reaction was started by addition of 20 μl methanol containing 800 nmole testosterone which had been recrystallised three times from warm acetone/water just prior to use. After 10 min the reaction was stopped by addition of 200 μl perchloric acid (12 M). Blanks, to which the substrate was added after stopping the reaction, were carried through the same procedure. Internal standard (40 μl of methanolic solution containing 1 μg 1-dehydrotestosterone) was added to all tubes which were then centrifuged (1500 *g*; 15 min; 4°). The supernatant was removed to fresh tubes and could be stored under liquid nitrogen for at least one month without any significant change in composition (see Results). Samples were thawed just prior to analysis, recentrifuged as before and 450 μl was injected onto the liquid chromatograph. The instrument used was a Waters Associates M6000A pump in conjunction with a U6K injector and 440 Absorbance Detector and was fitted with a 5 cm Co-pell octadecylsilane guard column and a 25 cm × 4.5 mm (i.d.) 5 μm Spherisorb octadecylsilane analytical column (both from Jones Chromatography, Llanbradach, Glamorgan, Wales). Elution was achieved at room temperature using a pressure ~ 750 p.s.i. and a flow rate of 0.5 ml/min 60:40 0.2 M sodium formate/1% formic acid : acetonitrile and detection was at 254 nm. Before and during continual use the column was conditioned overnight with 1% formic acid. No additional treatment was necessary between samples run on the same day. Standard curves for testosterone oxidation products were constructed by separately chromatographing several mixtures of different proportions of the authentic standards with a constant amount of internal standard (1-dehydrotestosterone). The peak heights on the resulting chromatogram trace were measured and the ratio of the reference peak height relative to that of the internal standard was plotted against the amount of reference compound used.

RESULTS

Assay of testosterone hydroxylations by high performance liquid chromatography. Figure 1 shows the profile of metabolites obtained using the high performance liquid chromatography method to analyse a mixture of reference compounds known to result from the monooxygenation of testosterone [11]. 1-Dehydrotestosterone was included as an internal standard. There was complete separation from testosterone of androstenedione and 7α-, 16α-, 6β- and 16β-hydroxytestosterones. Separation of 2α- and 2β-hydroxytestosterones was also achieved, although this was not complete if large amounts of both stereoisomers were present. An additional peak, eluting immediately before 7α-hydroxytestosterone, consisted of unseparated 6α- and 15α-hydroxytestosterones. Total elution time using the conditions described was around 60 min although this may vary depending on pressure and

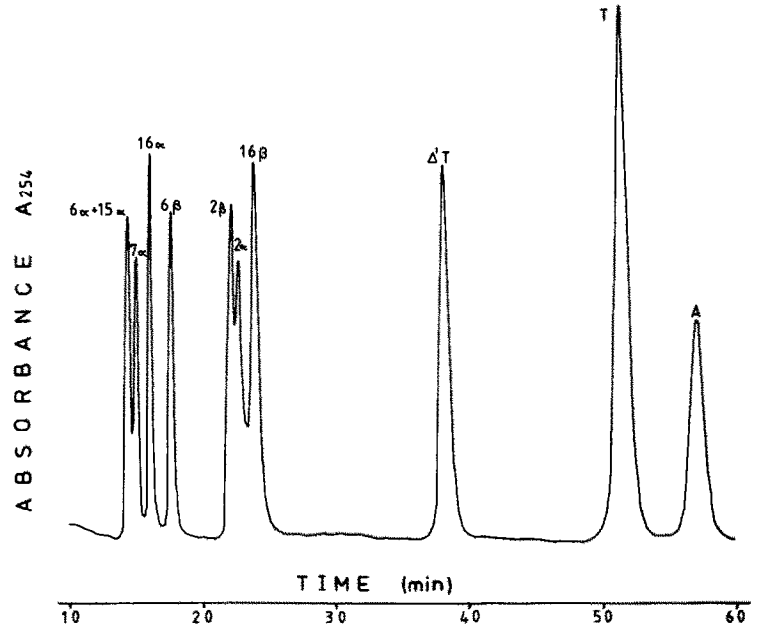

Fig. 1. Chromatogram of testosterone metabolites separated by the high performance liquid chromatography method described in the text. The peaks relate to standards of 2α-, 2β-, 6α-, 6β-, 7α-, 15α-, 16α- and 16β-hydroxytestosterones (denoted by numerical designation alone) and to testosterone (T), androstenedione (A) and the internal standard, 1-dehydrotestosterone (Δ^1T).

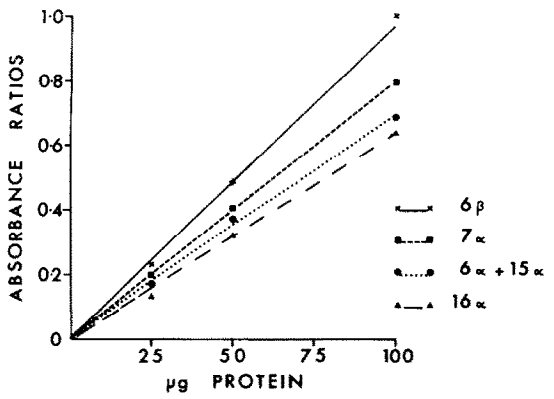

Fig. 2. Relationship between protein concentration and production of the major testosterone metabolites in microsomal incubations. Using the conditions and components described in Methods, incubations were performed in duplicate containing 25, 50 or 100 μg hepatic microsomal protein in 500 μl total volume. The production of 6α- + 15α-, 7α-, 6β- and 16α-hydroxytestosterones was quantitated as described in the text. Values are presented as the absorbance ratio of the peak height of the appropriate component to that of the internal standard and are denoted by the position of the hydroxylation catalysed.

the interval since the previous formic acid reactivation. The internal standard, 1-dehydrotestosterone, eluted around 38 min and was well separated from any testosterone-derived reference compound or from any major metabolite arising in the incubations we have so far performed.

Only testosterone and 1-dehydrotestosterone were detectable in incubations in which internal standard and substrate were added after stopping the incubation with perchloric acid. Repeated analysis of mixtures of internal standard and eight reference compounds (2α-, 2β-, 6α-, 6β-, 7α-, 15α-, 16α-, and 16β-hydroxytestosterones) dissolved in buffer showed coefficients of variation ranging from 1.67% (7α-hydroxytestosterone) to 5.37% (6β-hydroxytestosterone). No significant qualitative or quantitative differences in composition or chromatographic characteristics were detected when the same reference compounds were added to blank incubation mixtures, with recoveries ranging from 90.3% (6β-hydroxytesterone) to 103.9% (6α- + 15α-hydroxytestosterones). Similarly, after storage of post-incubation supernatants containing reference compounds in liquid nitrogen no significant changes in identity nor losses of metabolites were detected and recoveries ranged from 100.8% (2-hydroxytestosterones) to 106% (6β-hydroxytestosterone) and coefficients of variation from 0.83% (6α- + 15α-hydroxytestosterone) to 1.97% (6β-hydroxytestosterone). Using the conditions described, retention times of reference compounds relative to that of the 1-dehydrotestosterone internal standard (1.0) were 6α- + 15α-hydroxytestosterones: 0.376; 7α-hydroxytestosterone: 0.39; 16α-hydroxytestosterone: 0.44; 6β-hydroxytestosterone: 0.46; 2β-hydroxytestosterone: 0.56; 2α-hydroxytestosterone: 0.58; 16β-hydroxytestosterone: 0.63; testosterone: 1.35 and androstenedione: 1.50.

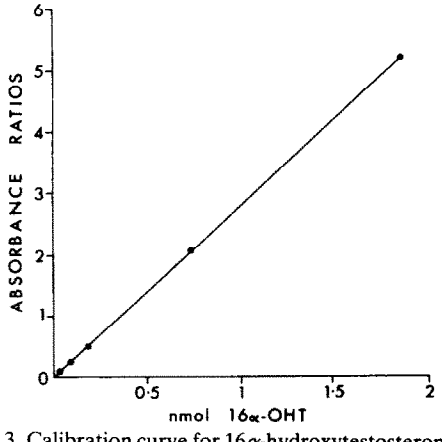

Fig. 3. Calibration curve for 16α-hydroxytestosterone (16α-OHT). The standard curve was prepared as described in the text following injection onto the chromatograph of differing amounts of 16α-OHT and a constant amount of internal standard. Data are presented as absorbance ratios of peak heights of 16α-OHT to the internal standard.

Addition of substrate in methanol had no significant effect on the qualitative or quantitative profile of testosterone oxygenation products. Following direct injection of a post-incubation supernatant, the elution profile was superimposed on the residual tail of the peak of polar incubation components (particularly $NADP^+$/NADPH) which eluted first from the column, e.g. Fig. 4. This effect could be minimised by reducing the microgram ratio of protein: $NADP^+$ to 1:1, a manipulation which had no effect on relative or absolute enzyme activities. Where measurements of peak heights had to be made from the sloping baseline this had no effect on the accuracy of the method. Accurate quantitation was possible and proportionality to protein concentration was achieved even using small amounts (25–100 μg) of microsomal protein (Fig. 2). In the example shown mouse microsomes were used and the four major metabolic products were determined.

Peaks were identified using visual inspection of chromatogram characteristics, relative retention times and by addition of reference compounds to duplicate incubations before chromatography. In subsequent discussions, peaks identified by these three criteria will be referred to as the co-chromatographing peak, but it must be emphasised that identifications are not absolute. Standard curves for 6β-, 7α-, 16α- and 16β-hydroxytestosterones and androstenedione were obtained as already described and showed proportionality over a wide range of concentrations. Correlation coefficients obtained by linear regression analysis exceeded 0.99 in all cases and a typical result is shown in Fig. 3 using 11.3—563 ng (0.037–1.85 nmole) 16α-hydroxytestosterone.

Effect of enzyme induction on microsomal testosterone monooxygenations. Typical testosterone metabolite profiles using microsomes from control and rifampicin-pretreated hamsters are shown in Fig. 4. The principal metabolite produced by control hamster liver microsomes (Fig.4a, peak 2) identifies as 7α-hydroxytestosterone by our criteria. Other major metabolites co-chromatographed with 6β-hydroxytestosterone (peak 5) and 6α- + 15α-hydroxytestosterones (peak 1). A 2-hydroxytestosterone isomer (peak 6) was produced which

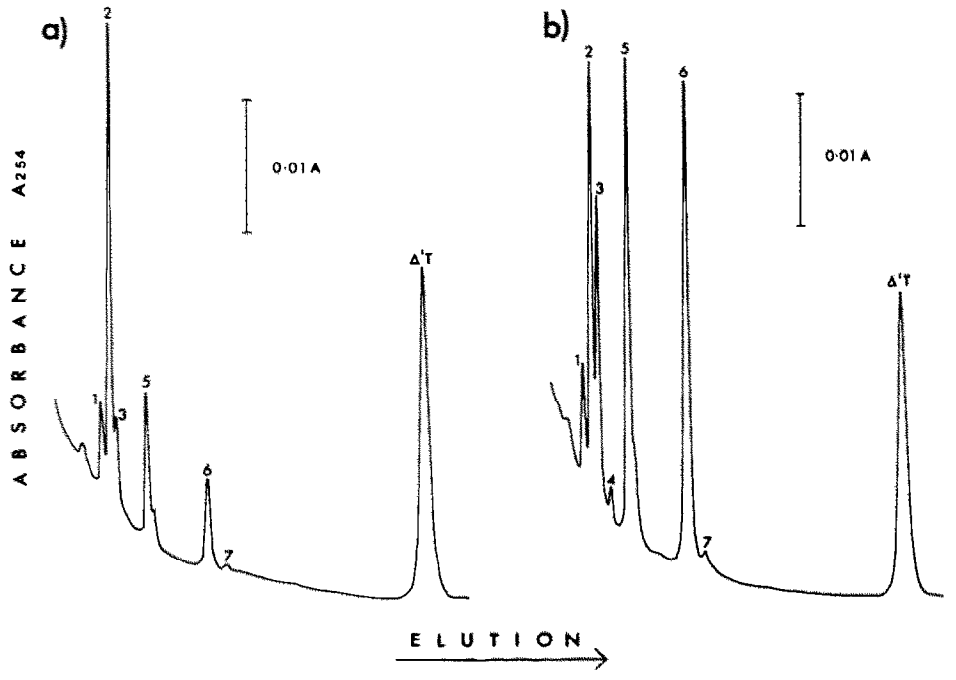

Fig. 4. Products of testosterone monooxygenation in incubations containing hepatic microsomal protein from (a) saline- or (b) rifampicin-pretreated hamsters. Shown in the figure are facsimiles of the chromatograms obtained using the assay methods described in the text. The peaks co-chromatographed with: 1: 6α + 15α-hydroxytestosterone (OHT); 2: 7α-OHT; 3: unidentified product; 4: 16α-OHT: 5: 6β-OHT; 6: 2β-OHT; 7: 16β-OHT; Δ¹ T: 1-dehydrotestosterone (internal standard).

our identification criteria suggested to be the 2β stereoisomer. There were small amounts of 16β-hydroxytestosterone (peak 7), 16α-hydroxytestosterone and androstenedione (peaks not shown) and these metabolites were quantitated using an absorbance range more sensitive than shown on the figure.

The marked changes in the profile of hepatic microsomal testosterone metabolites resulting from pretreatment of hamsters with rifampicin is depicted in Fig. 4b. Four to five-fold increases in the production of 6β-hydroxytestosterone (peak 5) are obvious as well as in the amounts of 2-hydroxytestosterones (peak 6). In addition, the production of an unidentified metabolite (peak 3) eluting immediately after 7α-hydroxytestosterone was substantially induced by rifampicin pretreatment as was 16α-hydroxytestosterone (peak 4). In contrast, the 7α-hydroxylation of testosterone (peak 2) was not substantially affected by rifampicin pretreatment and the amounts of 16β-hydroxytestosterone (peak 7) and androstenedione (not shown) were also unchanged.

The quantitative changes in metabolite profile following pretreatment with rifampicin, phenobarbitone, β-naphthoflavone and pregnenolone 16α-carbonitrile are compared in Fig. 5 for 6 β-, 7α-, 16α- and 16β-hydroxytestosterones. Phenobarbitone stimulated the 6β- and 16α-hydroxylation of testosterone 1–2-fold but after rifampicin these activities increased by 3–4-fold. No significant effect on 16β-hydroxylation was seen following pretreatment with phenobarbitone, rifampicin, pregnenolone 16α-carbonitrile or β-naphthoflavone, but β-naphthoflavone decreased testosterone 6β- and 7α-hydroxylations by 83 and 50%, respectively. The net result of these changes is that the profile of

Table 1. Changes in the *in vitro* production of microsomal testosterone metabolites following enzyme induction

Pretreatment	Absorbance ratio			
	6α + 15α	RR_t 0.405	2α/2β	A
Saline	0.22 ± 0.02	0.16 ± 0.02	0.26 ± 0.03	< 0.03
Rifampicin	0.25 ± 0.04	0.83 + 0.08*	1.4 ± 0.10*	< 0.05
Phenobarbitone	0.77 ± 0.06*	0.50 ± 0.02*	0.63 ± 0.06*	0.16 ± 0.01*
β-Naphthoflavone	0.77 ± 0.09*	< 0.05*	0.11 ± 0.01*	< 0.03
Pregnenolone 16α-carbonitrile	0.24 ± 0.03	0.22 ± 0.02	0.42 ± 0.03*	< 0.03

Hamsters were pretreated and enzyme assays performed as described under Materials and Methods. All results are the mean ± S.E. of 4 determinations and are presented as the absorbance ratios of the peak height of the appropriate component to that of the internal standard. The products considered are the 6α + 15α-hydroxytestosterone composite peak, an unidentified metabolite of relative retention time (RR_t) 0.405, the 2α/2β-hydroxytestosterone peaks and androstenedione.

* Significantly different from the saline pretreated control ($P < 0.05$; Student's *t*-test).

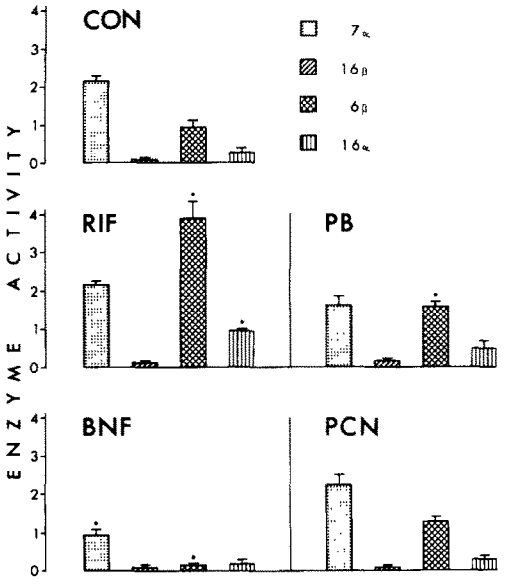

Fig. 5. Changes in the *in vitro* production of the major microsomal hydroxytestosterones following pretreatment of hamsters with enzyme inducing agents. Testosterone hydroxylase activities are denoted by the position of the hydroxylation catalysed and were determined as described in the text using hepatic microsomes from hamsters pretreated with saline (CON), rifampicin (RIF), phenobarbitone (PB), β-naphthoflavone (BNF) or pregnenolone 16α-carbonitrile (PCN). Enzyme activities are expressed as nmole hydroxytestosterone produced/mg microsomal protein/min (or per 10 min for 16β-hydroxytestosterone). Each value is the mean ± S.E. of four separate determinations. Asterisks denote results significantly different from the saline-pretreated control value ($P < 0.05$; Student's *t*-test).

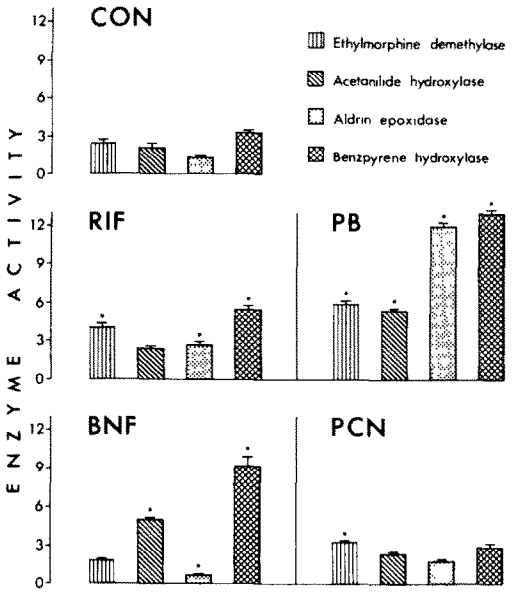

Fig. 6. Changes in *in vitro* xenobiotic enzyme activities following pretreatment of hamsters with enzyme inducing agents. Enzyme assays were performed as described in the text using liver microsomes from hamsters pretreated with saline (CON), rifampicin (RIF), phenobarbitone (PB), β-naphthoflavone (BNF) or pregnenolone 16α-carbonitrile (PCN). Enzyme activities are expressed as nmole product/mg microsomal protein/min or, for benzo[α]pyrene hydroxylation, RF units/mg microsomal protein/min [7]. Each value is the mean ± S.E. of four separate determinations with asterisks denoting values significantly different from the saline-pretreated control ($P < 0.05$; Student's *t*-test).

metabolites following induction with either rifampicin, phenobarbitone, or β-naphthoflavone is quite different from that found using microsomes from control hamsters. While pregnenolone 16α-carbonitrile pretreatment results in a profile of 6β-, 7α-, 16α- and 16β-hydroxytestosterone metabolites different from that following pretreatment with other enzyme inducers, the profile of these four metabolites is not significantly different from that found in control preparations.

Changes in several other incubation products of testosterone microsomal incubations are shown in Table 1. Quantitation has been made on the basis of peak height relative to the internal standard since the peaks concerned relate to minor metabolites or to unidentified or incompletely separated reaction products and extinction coefficients vary from one metabolite to another and cannot be assumed. Pretreatment with either phenobarbitone or β-naphthoflavone increased the magnitude of the peak comprising both 6α- and 15α-hydroxytestosterones but rifampicin or pregnenolone 16α-carbonitrile had no significant effect. Testosterone 2-hydroxylation was substantially increased by pregnenolone 16α-carbonitrile, rifampicin and phenobarbitone, but decreased by β-naphthoflavone. The magnitude of an unidentified peak (RR_f 0.405) eluting between 7α- and 16α-hydroxytestosterones (peak 3 in Fig. 4) was also decreased by β-naphthoflavone pretreatment but increased by rifampicin (5-fold), phenobarbitone (3-fold) and pregnenolone 16α-carbonitrile (1.4-fold). Only phenobarbitone pretreatment significantly increased androstenedione production.

Effect of enzyme induction on microsomal monooxygenation of exogenous substrates and related parameters. Relative to control values, pretreatment with either rifampicin or phenobarbitone increased hepatic microsomal protein yields (by 25 and 42%, respectively), but rifampicin decreased (by 19%) whereas phenobarbitone increased (by 10%) relative liver weight. The microsomal content of cytochrome P-450 in control animals (1.03 ± 0.04 nmole/mg microsomal protein; mean ± S.E., N = 4) increased by 32, 61 and 39% respectively following pretreatment with rifampicin, phenobarbitone and β-naphthoflavone, but pregnenolone 16α-carbonitrile had no significant effect. Only phenobarbitone (33% increase) and β-naphthoflavone (18% decrease) significantly affected NADPH-cytochrome *c* reductase activities (control rates: 174 ± 1.5 nmole cytochrome *c* reduced/mg microsomal protein/min; mean ± S.E., N = 4).

The effects of pretreatment with enzyme inducing agents on the monooxygenation of the four exogenous substrates ethylmorphine, acetanilide, aldrin and benzo[α]pyrene are shown in Fig. 6. The overall profiles of enzyme activities obtained after pretreatment with the four enzyme inducers are each dif-

ferent from that found in control microsomes. Relative to control values the only decreases in activity were for ethylmorphine demethylase and aldrin epoxidase and followed β-naphthoflavone pretreatment. In contrast, β-naphthoflavone increased threefold the oxygenation of both acetanilide and benzo[α]pyrene. Increases in all four enzyme activities followed phenobarbitone pretreatment and these ranged in magnitude from ~ two to ~ nine-fold for ethylmorphine demethylation and aldrin epoxidation, respectively. These same two activities were increased by about one-third following pretreatment with pregnenolone 16α-carbonitrile, but there was no significant effect on acetanilide or benzo[α]pyrene hydroxylations. While rifampicin pretreatment also caused no significant change in acetanilide hydroxylase activity, it increased the remaining three enzyme activities 1.5–2-fold.

DISCUSSION

In its present form the high performance liquid chromatography assay for testosterone metabolites we have developed provides a convenient direct method for analysis of the products of microsomal testosterone monooxygenation. It has several advantages over the conventional assays which involve the thin-layer chromatographic separation of radiolabelled testosterone metabolites extracted into organic solvent [10, 24] or the determination of tritium released during hydroxylation at a specific position on the steroid molecule [25]. Not least is the ability to compare simultaneously and quantitatively the production of multiple discrete peaks on the chromatogram following microsomal incubations with testosterone. Such measurements cannot be routinely achieved by thin-layer chromatography because of difficulties in identifying small amounts of products whose separation is limited by the inherent inadequacies in resolution of the system. Another major advantage is the ability to inject the incubation products directly onto the chromatograph without any requirement for solvent extraction. By so doing it is possible to avoid the sigmoidal kinetics resulting from incomplete dissociation of testosterone metabolites from small amounts of microsomal protein at the neutral pH required for solvent extraction from incubations*. Under the acidic conditions employed in the direct assay, such dissociation is complete and reaction linearity is maintained using as little as 25 μg microsomal protein. This level of sensitivity is approx. twice that we have achieved using the solvent system of Newton *et al.* [14] applied to a conventional column (rather than the radially compressed column described in the original method). It apparently results from the higher extinction coefficients of the testosterone metabolites in our own solvent system and enables the method to be applied to very small amounts of tissue.

Unlike the method of Van der Hoeven [11] and Wood *et al.* [13] the present assay employs isocratic elution and is therefore suitable for a liquid chromatograph fitted with a single pump assembly. Together with the need for a conventional 25 cm reverse phase octadecylsilane column and a 254 nm fixed wavelength ultraviolet detector these represent minimal requirements for liquid chromatography, although they may represent more exacting demands than for the thin-layer chromatography assay. One disadvantage of the method is the long elution time but this can be offset against the tedious and time-consuming steps for extracting, concentrating, plating, scraping off and counting samples in the thin-layer method. Other problems, relating to metabolite identification are typical of any liquid chromatography method where co-chromatography of known and unidentified products has always to be considered, and are again no greater than in the thin-layer assay. Moreover, where absolute identity is in doubt the overall profile of testosterone oxygenation products can provide valuable information which means that absolute identification is not imperative, although obviously useful.

Of the two major applications for the method, the more basic is to the detailed examination of testosterone metabolism in tissue subfractions. In the present study we have used liver microsomes isolated from hamsters because preliminary reports suggest this species is responsive to the effects of rifampicin [26] and we are interested in the experimental [7] and clinical interactions [27] of the antibiotic with steroid metabolism and therapy. Our present results show that the types and amounts of metabolites produced in control hamster liver microsomes are characteristically different from those already reported in rats [9], mice [28], rabbits [24], and man [29] using thin-layer chromatography assays and from the more extended profiles obtained in rats [11, 13*] or in mice, guinea pigs, rabbits or man* using liquid chromatography. Such variations in activity are typical of any group of mixed-function oxidase enzymes whose activities are compared in different animal species [30]. It is likely that they principally reflect the constitutive types of cytochrome P-450 present in the various preparations and the relative ability of these isoenzymes to catalyse the different enzymic reactions. The dissimilar responses of these different forms of cytochrome P-450 to enzyme inducing agents [31] introduces additional characteristic changes in microsomal testosterone hydroxylations in different animal species following enzyme induction. This is highlighted by differences in the effects of phenobarbitone, β-naphthoflavone, pregnenolone 16α-carbonitrile and rifampicin in hamsters presented here and those reported elsewhere using rats [11–13, 32, 33], rabbits [24], mice [7] and pigs [26]. Such comparisons clearly show that changes characteristic to one species cannot be generally assumed and are well exemplified by the increase in testosterone 7α-hydroxylation following polycyclic hydrocarbon pretreatment in rats [13, 32] but not in hamsters.

A second major application of the method was apparent when the extent of the heterogeneous response of different testosterone hydroxylations to enzyme inducing agents was demonstrated. As discussed above, such heterogeneity reflects the multiplicity of cytochromes P-450 and has been widely monitored by screening the total amount and spectral

* J. M. Tredger, H. M. Smith and R. Williams, unpublished observations.

characteristics of cytochrome P-450 and the activities of selected monooxygenases using exogenous substrates [7, 31, 34, 35]. Regiospecific testosterone hydroxylations have been used as an adjunct to this characterisation process [2, 5–8] but their value has been limited by the number of different metabolites which could be accurately quantitated. The more comprehensive evaluation of testosterone metabolism which the liquid chromatography assay permits may overcome this limitation. For example, using either exogenous substrates or testosterone a profile of enzymic reaction products was obtained which allowed discrimination between the effects on microsomal monooxygenases of rifampicin and three commonly used enzyme inducing agents. Our subsequent investigations* show that analagous differences in the monooxygenation of testosterone and xenobiotics prevail in male and female rat liver microsomes and following treatment of rats with ethanol and spironolactone. Consequently, comprehensive analysis of testosterone metabolites by high performance liquid chromatography may itself provide an effective means of categorizing enzyme inducing characteristics and identifying types of enzyme inducing agent. Therefore, by determining the activities of multiple testosterone hydroxylases in a single metabolic assay it is possible to obtain a broad expression of the catalytic potential of cytochromes P-450 isolated either as single purified forms or as heterogeneous mixtures in tissue preparations. The high sensitivity of the liquid chromatography method permits such determinations in very small amounts of microsomes and we have already made such measurements using fractions isolated from 5 to 10 mg portions of human liver biopsies. Additional improvements in separation, possibly using gradient elution, should extend the effectiveness of the assay and may permit its application in measurements of urinary metabolites of testosterone. It is envisaged that this approach could offer a non-invasive means of assessing drug metabolizing capacity analagous to that using 6β-hydroxycortisol [36] but with the additional benefit of permitting an assessment of the heterogeneous types of cytochrome P-450 present *in vivo*.

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