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A HIGHLY SENSITIVE TOOL FOR THE ASSAY OF CYTOCHROME P450 ENZYME ACTIVITY IN RAT, DOG AND MAN

DIRECT FLUORESCENCE MONITORING OF THE DEETHYLATION OF 7-ETHOXY-4-TRIFLUOROMETHYLCOUMARIN

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Abstract—The O-deethylation of 7-ethoxy-4-trifluoromethylcoumarin (EFC) by liver microsomes has been assessed as a method for monitoring the activity of cytochrome P450. The principle advantage of this substrate is the formation of a fluorescent product 7-hydroxy-4-trifluoromethylcoumarin (HFC) which can be assayed directly in the reaction medium. For rat microsomes the deethylated product was confirmed as the main metabolite, the reaction rate was linear with respect to both time and microsomal protein concentration and was independent of small changes in the added co-factors. A linear formation rate for the deethylated metabolite was also confirmed with dog and human microsomes. The intra-assay precision for rat, dog and human microsomes was 3, 5 and 4%, respectively. Hanes transformations of the dog and human data showed two phases, in contrast to a linear decline seen for the rat. Hybrid parameters for V_{max} and K_m, calculated from the apparently linear portions of these curves, gave inter-day SD for the V_{max} of rat, dog and man of 2, 14 and 4%, respectively, and approximately 15% for the K_m in all species. The V_{max} in rat, dog and human microsomes was 1.4 ± 0.2, 4.3 ± 1.5 and 0.9 ± 0.5 nmol HFC/min/nmol P450, respectively. The K_m was 11.0 ± 3.1, 67 ± 19 and 6.8 ± 2.5 μM, respectively. Direct evidence that at least two isoenzymes (cytochrome P450 1A2 and 2E1) metabolize EFC was obtained by experiments with competitive, suicide and immuno-inhibitors. Compared with ethoxycoumarin, the involvement of P450 2E1 in O-deethylation seemed similar in the rat. In conclusion, EFC provides a straightforward and reproducible assay for microsomal enzyme activity, requiring at most 25 pmol/mL of cytochrome P450.

In vitro tests for drug metabolism are used widely. As fresh animal and human material is not always available, samples are frequently stored at low temperatures, but the effect of storage is not well defined [1, 2]. In our department we use liver microsomes, a crude preparation of cytochrome P450, stored in liquid nitrogen. To test the effect of storage on the enzyme activity and as a daily control reaction for our microsomal incubations, we required marker reactions of cytochrome P450 activity. Such reactions should be easy to use, rapid and sensitive. This excluded the standard test substrates such as aminopyrine [3, 4] and ethoxycoumarin [5] which are confounded by work-up procedures or long analysis times.

7-Ethoxy-4-trifluoromethylcoumarin (EFC‡) has been described in the literature as an easy and sensitive method [6]. EFC is an analog of ethoxycoumarin, a widely employed cytochrome P450 test substrate. The reaction studied is given in Fig. 1. The fluorescence emission spectrum of the product, 7-hydroxy-4-trifluoromethylcoumarin (HFC), is different from those of EFC and NADPH (a required co-factor); therefore, the production of the metabolite can be monitored directly in the cuvette. Induction experiments showed a different cytochrome P450 pattern for EFC metabolism than for ethoxycoumarin [6], making both methods complementary. We therefore decided to evaluate this method for its usefulness as a daily control reaction and as a marker reaction for long term stability of microsomal preparations.

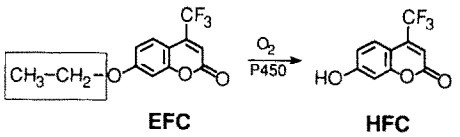

Fig. 1. The cytochrome P450 mediated O-deethylation of EFC to HFC.

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‡ Abbreviations: EFC, 7-ethoxy-4-trifluoromethylcoumarin; HFC, 7-hydroxy-4-trifluoromethylcoumarin; DMSO, dimethyl sulfoxide.

MATERIALS AND METHODS

Materials

EFC and its deethylated derivate, HFC, were

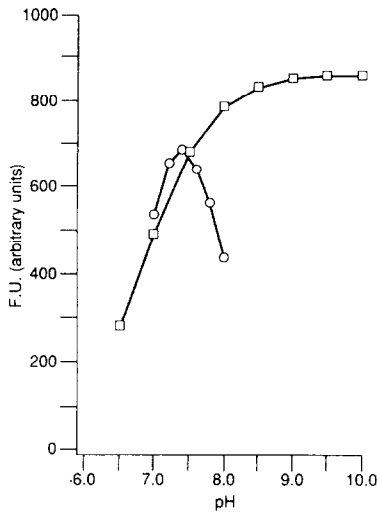

Fig. 2. The pH-dependent HFC formation by microsomes and HFC fluorescence in buffer solution. Formation of HFC by rat microsomes (○) and fluorescence of HFC in 0.1 M phosphate buffer without microsomes (□) as a function of pH. The single point EFC *O*-deethylase assay (N = 3) as described in Materials and Methods was used at 40 μM EFC.

obtained form Enzyme Systems Products (Dublin, CA, U.S.A.). Glucose-6-phosphate and glucose-6-phosphate dehydrogenase were from Boehringer (Mannheim, Germany). NADP, NADPH and α-naphthoflavone were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). The inhibitors 1- and 2-ethynylnaphthalene, 9-ethynylphenanthrene, 5-phenyl-1-pentyne and phenethylisothiocyanate were a kind gift from Prof. Dr W. L. Alworth, Tulane University (New Orleans, LA, U.S.A.). The immuno-inhibitive polyclonal antibodies anti-rat $P450_{ISF-G}$ (P450 1A2) and goat anti-rabbit $P450_{LM3a}$ (P450 2E1) were kindly supplied by Dr R. Peter, Biocenter (Basel, Switzerland) and Prof. M. J. Coon, Michigan University (Ann Arbor, MI, U.S.A.), respectively. Ethanol and dimethyl sulfoxide (DMSO) of Lichrosolv^R quality and TLC plates (art. 5729) were from Merck (Darmstadt, Germany).

Microsome preparation

Male albino rats [Lbm:RORO (SPF), 280–300 g, about 3 months, Biomedical Research Laboratories, Füllinsdorf, Switzerland] were killed by cervical dislocation and male beagle dogs (12–22 kg, about 11 years) were anesthetized with 30 mg/kg pentobarbital. The livers were immediately perfused with ice-cold 0.9% NaCl (20 mL for rats and 600 mL for dogs). The livers were excised, washed in ice-cold saline and quickly frozen in liquid nitrogen as small pieces. All livers were stored at −80°. Samples of human liver microsomes were kindly supplied by the Faculty of Pharmacy, Marseilles University, France. They were from healthy donors who were not elected for transplantation (mainly victims of traffic accidents, without liver disease). Microsomes (including human) were prepared according to standard procedures. In brief, the livers were homogenized in 4 vol. of 0.15 M KCl (pH 7.4) containing 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 0.02 mM butylated hydroxytoluene using 20 sec polytron homogenizing at 5000 rpm and three strokes of a Potter–Elvehjem homogenizer at 800 rpm. The homogenate was centrifuged for 15 min at 12,000 *g*, and then the supernatant was centrifuged for 15 min at 27,000 *g* and 1 hr at 105,000 *g*. The pellet was resuspended in 0.1 M sodium pyrophosphate buffer pH 7.4 containing 1 mM EDTA and centrifuged again for 1 hr at 105,000 *g*. The pellet was resuspended in 0.1 M phosphate buffer pH 7.4 and stored as 0.5 mL aliquots in liquid nitrogen until use.

Protein was determined by the method of Lowry *et al.* [7] using bovine serum albumin (BSA) as a standard. Cytochrome P450 was determined by the method of Omura and Sato [8] as modified by Rutten *et al.* [9].

Assays

Direct continuous EFC O-deethylase assay. EFC *O*-deethylase activity was determined according to the method of Deluca *et al.* [6]. In brief, 958 μL of 0.1 M phosphate buffer pH 7.4 containing 0.4 mg/mL bovine serum albumin was preheated to 37°, then 20 μL of microsomes diluted 1:10 in the same buffer and 20 μL of a NADPH regenerating system were added giving a final concentration of about 30 μg microsomal protein/mL, 0.5 mM glucose-6-phosphate, 0.1 mM NADP and 0.14 U/mL glucose-6-phosphate dehydrogenase. The reaction was started by adding EFC in 2 μL DMSO (0.25–100 μM) and stirring for 5 sec with a motor-driven handmixer for cuvettes (Hellma, Müllheim, Germany). A jacketed cuvette holder heated by a circulating waterbath kept the reaction at 37°. The increase of fluorescence with time was monitored on an Aminco SPF-500 TM fluorescence spectrophotometer with excitation at 410 nm (slit width 2 nm) and emission at 510 nm (slit width 10 nm). The reaction was allowed to proceed for 2 min, and the rate of HFC formation was calculated from the slope of a recorded trace. With each study a calibration curve with HFC in an identical reaction mix was performed. All determinations were done at least in duplicate.

Single point EFC O-deethylase assay. Alternatively, EFC O-deethylation was determined by incubating for 10 min at 37° in a waterbath. The reaction was stopped with ice-cold acetonitrile (final concentration 25%) and the fluorescence was determined at room temperature without any further work-up. This method was used for the time and pH dependency experiments.

7-Ethoxycoumarin O-deethylation by liver microsomes. This was determined according to Greenlee and Poland [5] using 0.1 M sodium borate pH 10 as a fluorescence buffer.

Formation of metabolites. This was examined by TLC, according to Deluca *et al.* [6]. Ten milliliters of 0.6 nmol cytochrome P450/mL were incubated with 100 μM EFC and the NADPH generating system for 20 min. The reaction was stopped with

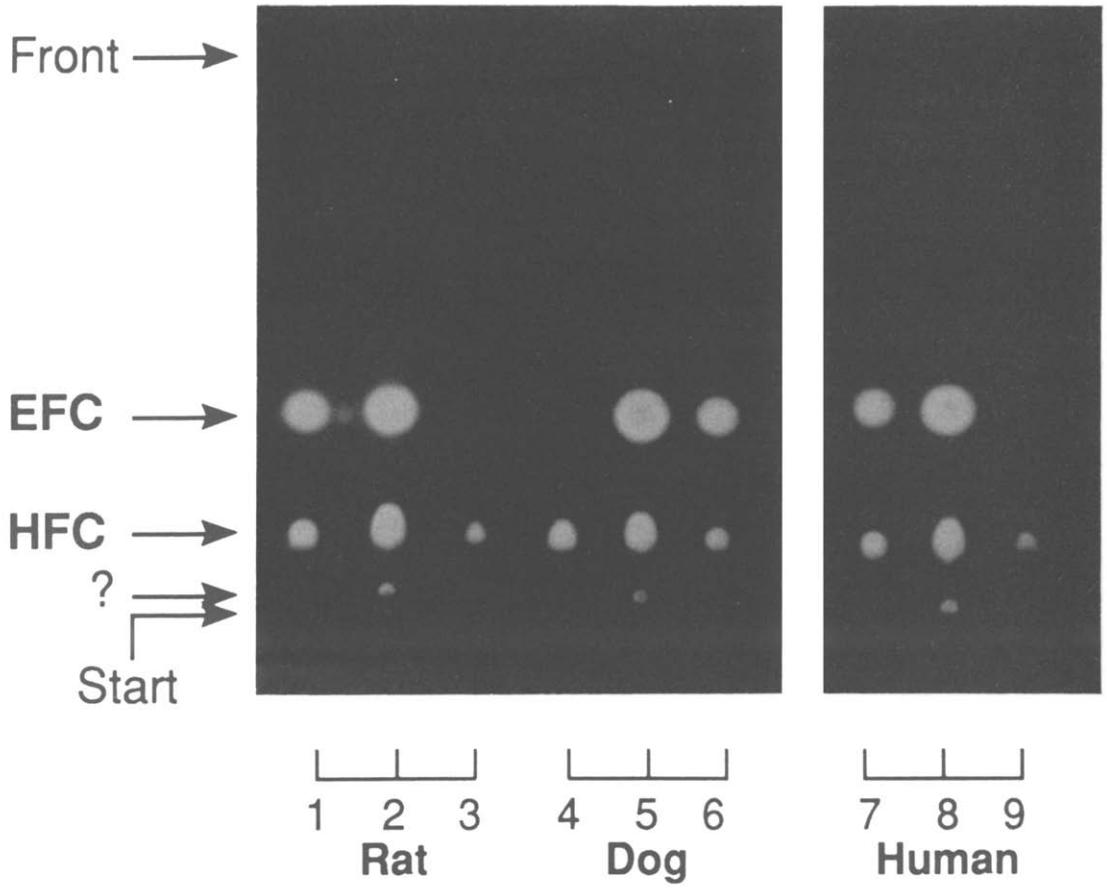

Fig. 3. Polaroid photographs of a TLC separation of EFC and its metabolites with UV detection. Liver microsomes of each species were incubated with either EFC (lanes 2, 5, 8) or HFC (lanes 3, 4, 9). Pure EFC and HFC were co-eluted on lanes 1, 6 and 7 (no incubation). The minor metabolite (marked ?) showed the same blue fluorescence as EFC. HFC had a green fluorescence.

3 mL 15% trichloracetic acid and extracted with 5 mL hexane–petroleum ether–ethylacetate (1:1:1). The organic solvent was evaporated to dryness and the residue redissolved in 20 μL MeOH. Samples were spotted on silica gel TLC plates and developed in hexane–ethylacetate (5:2). Developed plates were examined for fluorescence under 366 nm illumination.

The effect of inhibitors. Competitive inhibitors were tested by co-incubating them with EFC and microsomes as described above. Suicide inhibitors were pre-incubated with microsomes and the NADPH generating system for 30 min at 37°, then EFC and fresh NADPH generating system were added and the reaction was monitored as described above. The immuno-inhibitive antibodies were pre-incubated with microsomes as concentrated solutions at room temperature for 30 min and then preheated buffer was added. When the reaction reached 37°, EFC and the NADPH generating system were added. All inhibitors were dissolved in DMSO, except the antibodies. The reactions were performed at least in triplicate and each reaction had its own control under identical conditions. The immuno-inhibitive experiments with P450 2E1 were done in duplicate due to the large amounts of antibodies needed.

Statistical analysis

Differences between two groups were evaluated by either paired or unpaired Student's *t*-test [10]. Curves were compared with the paired Student's *t*-test. Differences between more than two groups were evaluated by Peritz' F-test [11]. $P < 0.05$ was considered statistically significant.

RESULTS

Stability of EFC and HFC

EFC dissolved in DMSO was reported to be stable for more than 1 year at −20° [6]. In short-term experiments we found that EFC samples stored in DMSO in the dark at +20° for 2 weeks gave 3–5% higher enzyme activity than samples stored at −80° at 100 μM (about V_{max}) and 10 μM (about K_m), confirming EFC stability.

Stability of HFC in DMSO was examined by

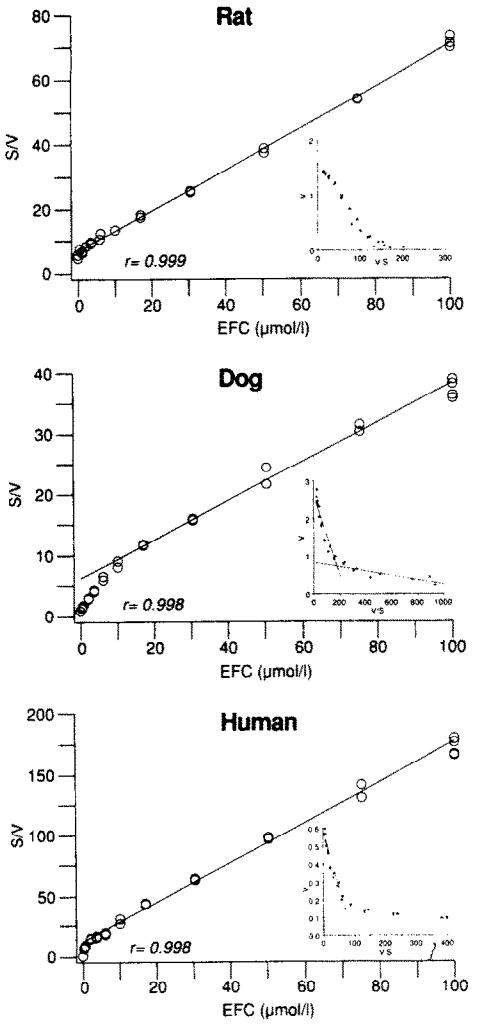

Fig. 4. Representative examples of Hanes and Eadie–Hofstee (inserts) transformations of EFC deethylation in liver microsomes of rat, dog and man. In the rat only one apparent phase of V_{max} and K_m could be seen. In dog and man two phases in the Hanes transformation were seen, being more pronounced in dog than in man. The regression equation for the rat was $Y = 0.631X + 6.69$, for dog $Y = 0.318X + 6.23$ and $Y = 1.675X + 12.62$ for man.

storing solutions in the dark at −80° and +20° for 2 months. Differences between the calibration curves were not noticed (−80° vs 2 months +20°, −0.7%, not significant, paired Student's *t*-test).

General characteristics of the reaction

Our preliminary studies confirmed the results presented by Deluca *et al.* [6]. This method is extremely sensitive, and we generally were able to use as little as 25 pmol cytochrome P450/mL. Diluting the microsomes did not affect the sensitivity of the assay, but a non-specific loss in fluorescence was seen. Again we have confirmed that this loss in fluorescence can be overcome by the addition of bovine serum albumin [6]. When our microsomes were diluted 1 to 10 with 0.1 M phosphate buffer pH 7.4 the activity per nanomole cytochrome P450 appeared to be reduced by 8.9% ($P < 0.001$, Peritz' F-test). However, addition of bovine serum albumin (18 μg/mL) to the buffer prevented this non-specific loss (fluorescence + 3.5%, not significant, Peritz' F-test). To reduce the amount of microsomes needed further we considered replacing the microsomes in the calibration curve by bovine serum albumin. When neither microsomes nor bovine serum albumin were used for the calibration curve, the measured microsomal activity was reduced by 7.7% ($P < 0.05$, paired Student's *t*-test). Bovine serum albumin only partially counterbalanced this effect and we therefore currently use the same concentration of microsomes for both the assay and calibration. When only a limited supply of material is available, however, bovine serum albumin could replace microsomal protein as the error is only about 3.5% ($P < 0.05$, paired Student's *t*-test). Changing the relative amounts of the NADPH generating system or replacing it with 0.2 mM NADPH had no influence on the rate of the reaction (data not shown). In contrast, DMSO inhibited the reaction by 11 ± 3% ($N = 5$) in rat and dog microsomes. This was not due to a quenching effect of DMSO on HFC fluorescence.

The dependency of the reaction and the fluorescence of the formed product on pH is shown in Fig. 2. As for ethoxycoumarin the optimum pH for the fluorescence differs from the pH optimum for the microsomal reaction [5]. HFC shows as 7-hydroxycoumarin maximum fluorescence intensity at pH values > 9.5. However, the intensity at pH 7.4 (pH of microsomal incubation) is already sufficient for all determinations.

Reaction products

The reaction products formed by the microsomes of rat, dog and man were examined by TLC. To increase the sensitivity of detection, larger volumes with a total of 6 nmol cytochrome P450 were incubated. In all species HFC (green fluorescence) was the major reaction product (Fig. 3). Apart from the unchanged substrate (blue fluorescence) only one small fluorescent spot (blue fluorescence) was detected on the plates and staining with iodine vapour or H_2SO_4 did not reveal any non-fluorescent bands. For all three species, therefore, HFC appears to be by far the most abundant reaction product, and we could not detect any evidence for its further metabolism.

Linearity of reaction

The linearity of the reaction was tested with the single point method. The rate of reaction was linear up to at least 30 min in all species with 100 μM EFC (the highest concentration). Stirring of the direct continuous assay did not change the rate of formation of the metabolite.

Assay kinetics

The enzyme kinetics of EFC deethylation by rat, dog and human microsomes were determined in four experiments for each species. Typical results are shown in Fig. 4. In the rat only one phase of V_{max}

Table 1. The mean V_{max} and K_m of EFC O-deethylation of rat, dog and human microsomes

Species	V_{max} (nmol HFC/min/nmol P450)	K_m (μM)
Rat	1.39 ± 0.18	11.0 ± 3.1
Dog	4.25 ± 1.48	67 ± 19
Human	0.89 ± 0.49	6.8 ± 2.5

Means ± SD are given.

and K_m was apparent from the linear decline in the Hanes and Eadie–Hofstee plots. For dog and human microsomes, however, two phases in the Hanes and Eadie–Hofstee plots were seen.

V_{max} and K_m were calculated from a linear regression equation fitted to the linear part of the Hanes transformed data. For rat the concentrations 0–100 μM (N = 13), for dog 10–100 μM (N = 6) and for man 1–100 μM (N = 11) were used for the fitting. For dog and man this approach yields hybrid parameters.

The intra-assay precision of EFC O-deethylation (100 μM) in rat, dog and man was 2.7, 5.2 and 4.0%, respectively (N = 6). The interday variability in V_{max} for rat, dog and man was 1.7, 13.5 and 4.4%, respectively (two batches for rat and dog and one for man, 3 days). For K_m these values were 14.7, 14.5 and 11.0%, respectively.

The V_{max} and K_m of rat, dog and human microsomes are shown in Table 1. Deluca *et al.* [6] reported for uninduced Sprague–Dawley rat microsomes a V_{max} of 1.84 nmol HFC/min/mg protein and a K_m of 4.15 μM.

Cytochrome P450 isoenzymes

EFC O-deethylation responded to both phenobarbital and methylcholanthrene induction [6]. To gain more insight into the cytochrome P450 isoenzymes catalysing the O-deethylation of EFC, inhibition studies were performed in uninduced rat microsomes. The classic inhibitor of the cytochrome 1A family (P450 1A1 and 1A2), α-naphthoflavone [12–14], inhibited EFC O-deethylation by 57%. 2-Ethynylnaphthalene, a specific mechanism-based inactivator of P450 1A2 [15, 16], was also found to inhibit the reaction (Table 2). A similar compound 1-ethynylnaphthalene, a competitive inhibitor of P450 1A2 [15], gave the same inhibition (29%). A novel compound of the suicide-type inhibitors, 9-ethynylphenanthrene with activity against P450 1A and P450 2B1 (Alworth, personal communication), reduced the formation of HFC by 58%.

Reactions catalysed by P450 1A2 often show cross-reactivity with cytochrome P450 2E1 (P450 2E1) as shown by acetaminophen [17] or aniline [18]. The metabolic hydroxylation of chlorzoxazone is catalysed selectively by P450 2E1 in human liver, thus it is considered to be a specific probe for this enzyme [19]. Co-incubation of EFC with

Table 2. The effect of inhibitors and antibodies against cytochrome P450 on the O-deethylation of EFC and 7-ethoxycoumarin (EC) by hepatic microsomes from uninduced rats

	Isoenzyme inhibited	Inhibitor concentration (μM)	Inhibition of EFC (%)	Inhibition of EC (%)
Competitive inhibition				
α-Naphthoflavone	1A2*	10	57 ± 1	−12 ± 2†
Chlorzoxazone	2E1	500	67 ± 1	47 ± 8
Suicide inhibition				
2-EN	1A2‡	2.5	29 ± 1	44 ± 2
9-EP	1A2*†‡	2.5	58 ± 4	67 ± 2
PEITC	2E1‡	2.5	62 ± 4	79 ± 1
5-PP	2E1‡§	2.5	57 ± 12	45 ± 2
Immuno-inhibition				
Anti-rat $P450_{ISF-G}$	1A2	20 mg/nmol P450	11 ± 3	ND
	1A2	80 mg/nmol P450	15 ± 3	ND
Anti-rabbit $P450_{LM3a}$	2E1	5 mg/nmol P450	42, 44	ND
	2E1	10 mg/nmol P450	45, 59	ND

The competitive inhibitors were co-incubated with 10 μM EFC or 100 μM EC (about K_m), rat microsomes and the NADPH regenerating system. The suicide inhibitors 2-ethynylnaphthalene (2-EN), 9-ethynylphenanthrene (9-EP), phenethylisothiocyanate (PEITC) and 5-phenyl-1-pentyne (5-PP) were pre-incubated with, and the antibodies without, the NADPH generating system (see Materials and Methods). DMSO inhibits the reaction by 11%. The column % inhibition is corrected for this effect. All assays were done at least in triplicate.

* Inhibits P450 1A1 and 1A2, the former being only present in low concentrations in uninduced microsomes.

† Stimulation of deethylation activity.

‡ Inhibits also P450 2B1, which is only present in low concentrations constitutively.

§ Personal communication, Prof. Dr W. L. Alworth and N. E. Hopkins.

ND, not determined.

Table 3. The activity of EFC O-deethylation by liver microsomes of different species

Species	Activity (nmol HFC/min/nmol P450)
Mouse	1.83 ± 0.03
Rat	1.41 ± 0.03
Rabbit	0.86 ± 0.03
Dog	2.69 ± 0.10
Squirrel monkey	0.41 ± 0.02
Cynomolgus monkey	2.14 ± 0.06
Human	0.58 ± 0.02

The activity of 100 μM EFC was measured (one batch per species, N = 4). This value is lower than the V_{max} in some species. Therefore these values do not necessarily represent V_{max}. Undiluted microsomes were measured and in the calibration curve bovine serum albumin was used instead of the different microsomal preparations.

chlorzoxazone inhibited the metabolic generation of HFC by 67%. Phenylisothiocyanate was recently shown to be a relatively specific suicide-type inactivator for P450 2E1 [20]. Another suicide-type inactivator of P450 2E1 (with some activity for P450 2B1) is 5-phenyl-1-pentyne (Alworth, personal communication). Both compounds inhibited the formation of HFC extensively (Table 2).

An immuno-inhibitive polyclonal antibody against P450 1A2, which inhibits this isoenzyme by 80% (Peter, personal communication), inhibited the reaction by 15%. A polyclonal antibody against P450 2E1 (maximal inhibition of this isoenzyme 85% [19]) inhibited the formation of HFC by 52%.

Testing several of these inhibitors with ethoxycoumarin O-deethylation showed a similar involvement of P450 2E1 (see Table 2, chlorzoxazone, phenethynylisothiocyanate and 5-phenyl-1-pentyne). α-Naphthoflavone, however, exerted a different effect on the two substrates.

Species differences

Microsomal preparations of the mouse, rat, rabbit, dog, squirrel, cynomolgus monkey and man were tested for EFC deethylation activity (Table 3). EFC O-deethylation was measured at 100 μM. This value was lower than the V_{max} in some species; therefore, these data do not necessarily represent V_{max}. Microsomes of human had a low activity and rodents a high activity for EFC O-deethylation. Cynomolgus monkey had a 3-fold higher activity than the other primates.

DISCUSSION

In our laboratories, we have been seeking to identify a reaction which can be used routinely to evaluate the stability of cytochrome P450 stored as microsomes in liquid nitrogen, and to monitor incubations for which we routinely use rat, dog and human microsomes. For this purpose we have studied the novel coumarin analog EFC. As the use of this reagent has only previously been described by Deluca *et al.* [6], we also needed to confirm and elaborate on some aspects of this reaction.

We were able to confirm that EFC provides a sensitive test reaction for cytochrome P450 enzyme activity. Although several similar reactions are available for cytochrome P450, such as ethoxycoumarin [5, 21] and the alkoxy resorufins [22, 23], and the less commonly employed 3-cyano-7-ethoxycoumarin [24], scoparone [25] and methoxycoumarin [26], EFC offers some distinct advantages.

In all species studied EFC was metabolized almost exclusively to HFC and only trace amounts of a second product could be detected (Fig. 3). This excludes invisible depletion of substrate. The amount of microsomal protein needed for an assay is low (25 pmol cytochrome P450/mL, about 30 μg protein/mL, or less) which makes it especially interesting when working with human material or hepatocytes where little cytochrome P450 is available [27]. The assay was reproducible and has the additional advantages that microsomal activity can be measured without sample clean-up. EFC and HFC were found to be stable and currently we store all components "ready for use" at −80°, allowing it to be used on a daily basis.

The reaction was linear up to 30 min under the conditions which we evaluated. The intra-assay variation was 5% or lower for all three species. The inter-day variation in V_{max} for rats and humans was below 5% but was 14% in the dog. For K_m the inter-day variation was approximately 15% in these species.

Deluca *et al.* [6] clearly showed that the deethylation of EFC is cytochrome P450 dependent. The isoenzymes responsible for EFC metabolism were unknown, a feature shared by many of the above-mentioned cytochrome P450 test reactions, especially when uninduced microsomes are used. In contrast to Deluca *et al.* [6] we found only one apparent V_{max} and K_m in RORO rats (Fig. 4). Nevertheless, we obtained evidence that more than one isoenzyme metabolized EFC. Thus, α-naphthoflavone, an inhibitor of P450 1A1 and 1A2 [12–14], inhibited the reaction (Table 2). The absence of inhibition of ethoxycoumarin O-deethylation by α-naphthoflavone was also reported by other authors [2]. P450 1A1 and 2B1 are only present at low levels in the uninduced rat liver [28, 29]. This indicates that P450 1A2 is responsible for part of the EFC deethylation. The suicide inhibitor of P450 1A2, 2-ethynylnaphthalene [15], and an immuno-inhibitive antibody against P450 1A2 confirmed the involvement of this isoenzyme.

More important was the involvement of P450 2E1. The reaction was inhibited by chlorzoxazone, a specific substrate of P450 2E1 [19]. The suicide inhibitors of P450 2E1, phenethylisothiocyanate [20] and 5-phenyl-1-pentyne, and an immuno-inhibitive antibody against P450 2E1 supported the involvement of this isoenzyme.

These results show that in the RORO rat strain, the O-deethylation of EFC is mediated to about 15% by P450 1A2 and about 60% by P450 2E1. For ethoxycoumarin the involvement of P450 2E1 as the main metabolizing cytochrome P450 isoenzyme was similar (about 60%). Deluca *et al.* [6] found little

effect of the P450 2E1 inducer ethanol, in disagreement with our results. The remaining enzyme activity indicates that other P450 isoenzymes may be involved. Deluca *et al.* [6] had earlier recorded no effect of the inducer dexamethasone, excluding a role of P450 3A. For ethoxycoumarin the involvement of human P450 2A6 (related rat protein: P450 2A1) was recently shown and this isoenzyme may also play a role in EFC metabolism [30].

With human and dog microsomes two phases in the Hanes plot provided direct evidence that at least two different isoenzymes were responsible for EFC deethylation (Fig. 4). Due to homology of P450 isoenzymes between species [13, 31–33] we would expect similar isoenzymes (i.e. P450 1A2 and 2E1) to be responsible for EFC metabolism.

The use of a direct continuous assay can also have the disadvantage that one has to wait for the reaction to be recorded (usually 2 min) before starting a new one. Fortunately, the method can be easily modified to provide a fast, single point method as the reaction can be stopped with acetonitrile. Longer incubations are then practical, thereby further increasing the sensitivity of the method. A disadvantage of the method is the low solubility of EFC in buffer, requiring the addition of 0.2% DMSO which inhibits the reaction by about 10%.

The results show that EFC offers some distinct advantages over other substrates in testing cytochrome P450 isoenzyme activity. The excellent reproducibility of the method makes it very suitable for testing long-term stability of microsomal preparations. The small amount of microsomes needed and the straight-forward analysis make this substrate more appropriate for routine use than ethoxycoumarin.

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