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Determination of cytochrome P450 1A activities in mammalian liver microsomes by high-performance liquid chromatography with fluorescence detection

Nobumitsu Hanioka[a,*], Norihisa Tatarazako[b], Hideto Jinno[a], Koji Arizono[c], Masanori Ando[a]

[a]*Division of Environmental Chemistry, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[b]*Department of Environmental Biology, Japan Pulp and Paper Research Institute, 5-13-1 Tokodai, Tsukuba 300-2635, Japan*
[c]*Faculty of Environmental and Symbiotic Sciences, Prefectural University of Kumamoto, 3-1-100 Tsukide, Kumamoto 862-8502, Japan*

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Abstract

A sensitive method for the determination of cytochrome P450 (P450 or CYP) 1A activities such as ethoxyresorufin *O*-deethylase (EROD) and methoxyresorufin *O*-demethylase (MROD) in liver microsomes from human, monkey, rat and mouse by high-performance liquid chromatography with fluorescence detection is reported. The newly developed method was found to be more sensitive than previous methods using a spectrofluorimeter and fluorescence plate reader. The detection limit for resorufin (signal-to-noise ratio of 3) was 0.80 pmol/assay. Intra-day and inter-day precisions (expressed as relative standard deviation) were less than 6% for both enzyme activities. With this improved sensitivity, the kinetics of EROD and MROD activities in mammalian liver microsomes could be determined more precisely. EROD activities in human and monkey liver microsomes, and MROD activities in liver microsomes from all animal species exhibited a monophasic kinetic pattern, whereas the pattern of EROD activities in rat and mouse liver microsomes was biphasic. In addition, the method could determine the non-inducible and 3-methylcholanthrene-inducible activities of EROD and MROD in rat and mouse liver microsomes under the same assay conditions. Therefore, this method is applicable to in vivo and in vitro studies on the interaction of xenobiotic chemicals with cytochrome CYP1A isoforms in mammals. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Cytochrome P450; Ethoxyresorufin; Methoxyresorufin

1. Introduction

Cytochrome P450 (P450 or CYP) enzymes are heme-thiolate proteins that are responsible for the oxidative metabolism of xenobiotic chemicals such as drugs and environmental chemicals, as well as endobiotic chemicals such as steroids, fatty acids and vitamins [1]. They comprise a superfamily of related enzymes that are grouped into families and subfamilies based on similarities in amino acid or nucleotide sequences [2]. Most of the xenobiotic chemicals have been shown to be catalyzed mainly by one to three families of P450s and individual

*Corresponding author. Tel.: +81-3-3700-1141; fax: +81-3-3707-6950.

E-mail address: hanioka@nihs.go.jp (N. Hanioka)

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P450 enzymes have considerable, but overlapping, substrate specificities [1,2].

The CYP1A subfamily includes two genes, *CYP1A1* and *CYP1A2*, which show a relatively high degree of nucleotide homology in mammals [1,2]. There are numerous interesting aspects to these two P450 isoforms. Although the induction mechanism is different between *CYP1A1* and *CYP1A2*, both of these P450 isoforms are remarkably induced by polycyclic aromatic hydrocarbons and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in experimental animals [1,3]. Therefore, the determination of *CYP1A1* and *CYP1A2* activities in mammals is an important aspect of toxicological research. Ethoxyresorufin *O*-deethylase (EROD) and methoxyresorufin *O*-demethylase (MROD) activities are now probably the most commonly used assays for *CYP1A1* and *CYP1A2*, respectively [4–7].

Most laboratories use an assay of EROD and MROD activities that is adapted from the spectrofluorimetric method developed by Pohl and Fouts [8] and by Burke et al. [4]. On the other hand, Kennedy et al. [9] have reported a method to determine EROD activity using a fluorescence plate reader. Since, as these methods suffer from low sensitivity and non-specific fluorescence, they do not allow for the determination of low-activity samples. Recently, an assay for EROD activity using high-performance liquid chromatography (HPLC) has been developed, but it requires an extraction step [10]. In addition, resorufin is formed from the substrate in the blank sample using liver microsomes.

In this study, we report a simple and sensitive method for the determination of EROD and MROD activities in liver microsomes from human, monkey, rat and mouse by HPLC with fluorescence detection.

2. Experimental

2.1. Materials

Resorufin (>99% pure), ethoxyresorufin (>99% pure) and methoxyresorufin (>98% pure) were obtained from Sigma (St. Louis, MO, USA). NADPH was a product of Oriental Yeast (Tokyo, Japan). Pooled liver microsomes from 10 human donors (16–73 years old) were purchased from Gentest (Woburn, MA, USA). Pooled liver microsomes from five male cynomolgus monkeys (3–5 years old) were obtained from In Vitro Technologies (Baltimore, MD, USA). Pooled liver microsomes from 10 male Sprague–Dawley rats (8 weeks old) and 30 male Swiss mice (8 weeks old) were prepared as previously described [11]. Liver microsomal preparations for induction studies using rat and mouse were performed as previously reported [11]. The microsomal protein content was determined according to Lowry et al. [12] using bovine serum albumin as standard. The total microsomal P450 content was determined according to Omura and Sato [13]. The microsomal samples were stored at −80°C until use. All other materials were of the highest quality commercially available.

2.2. Standards

A stock solution (4 m*M*) of resorufin was prepared by dissolving 18.8 mg of resorufine in 20 ml of methanol. Stock solutions (0.4–400 μ*M*) for calibration curves were prepared by serial dilutions of the 4 m*M* stock solution with methanol. These solutions were stored at 4°C protected from light and were stable for at least 1 month. Working solutions were prepared daily by diluting the stock solutions with 50 m*M* phosphate buffer (pH 7.4)–methanol (50:50).

2.3. Incubations

EROD and MROD activities were determined by quantification of the resorufin production from dealkylation of ethoxyresorufin and methoxyresorufin by liver microsomes. The standard incubation mixture contained ethoxyresorufin (15–2000 n*M*) or methoxyresorufin (25–1000 n*M*) each spiked separately as substrate, liver microsomal proteins from human, monkey, rat or mouse (0–300 μg) and 1 m*M* NADPH in a final volume of 500 μl of 50 m*M* phosphate buffer (pH 7.4). Both substrates were dissolved in methanol (final concentration in the reaction medium, 0.5%, v/v). After preincubation at 37 or 25°C for 1 min, the reaction was started by the addition of NADPH. The mixture was incubated at 37 or 25°C for 0–30 min and the reaction terminated with 500 μl of ice-cold methanol with vortexing.

After cooling on ice for 15 min, the samples were centrifuged at 6000 *g* for 20 min. The supernatant was filtered with a PTFE membrane filter of 0.45 μm pore size (Millipore, Bedford, MA, USA) and analyzed by HPLC immediately. The samples were stable for at least 5 days (at 4°C). Blank samples contained all components except the NADPH which was added after termination of the reaction.

2.4. Apparatus and HPLC conditions

HPLC analysis was performed using a Shimadzu SCL-10A system controller (Kyoto, Japan) consisting of three LC-10AD pumps, a SIL-10A autoinjector with sample cooler, a RF-550 fluorescence detector, a CTO-10A column oven, a DGU-3A degasser and a C-R4A chromatopac integrator. The samples (50 μl) were injected into an Inertsil ODS-80A column (5 μm, 150×4.6 mm I.D., GL Sciences, Tokyo, Japan). The column was kept at 40°C. The product was eluted isocratically with 20 m*M* phosphate buffer (pH 6.8)–methanol–acetonitrile (52:45:3, v/v) at a flow-rate of 0.8 ml/min. The excitation and emission wavelengths were fixed at 560 and 585 nm, respectively. The formation rates of product from ethoxyresorufin or methoxyresorufin were calculated from the peak areas of different concentrations of resorufin. Standards for resorufin were prepared from stock standard solutions at concentration of 1.0, 2.0, 5.0, 10, 20, 50, 100, 200, 500 and 1000 pmol/ml as described above.

2.5. Kinetic analysis

Incubation conditions were chosen such that product formation was linear with respect to both microsomal protein amount and incubation time for the determination of EROD and MROD activities in liver microsomes from all animal species. Microsomal protein amounts for the determination of EROD activity were: 60 μg in human; 15 μg in monkey; and 40 μg in rat and mouse. Microsomal protein amounts for the determination of MROD activity were: 60 μg in human; 40 μg in monkey and rat; and 25 μg in mouse. Substrate concentrations for the determination of EROD activity were: 50–2000 n*M* in human, rat and mouse; and 15–300 n*M* in monkey. Substrate concentrations for the determination of MROD activity were 25–1000 n*M* in all animal species. Incubation times for the determination of both enzyme activities were 6 min in all animal species. The Michaelis–Menten parameters such as K_m and V_{max} were estimated by analyzing Eadie–Hofstee plots using the software Enzyme-Kinetics v.1.4 (Trinity Software, Campton, NH, USA).

2.6. Induction study

EROD and MROD activities in control and 3-methycholanthrene-pretreated liver microsomes from rat and mouse were measured separately as described above. The reactions were performed with a substrate concentration of 1000 n*M* for EROD or 500 n*M* for MROD, and liver microsomal protein amounts of 15 μg at 25°C for 6 min.

3. Results and discussion

3.1. HPLC chromatograms

Liver microsomes from human, monkey, rat and mouse were incubated with ethoxyresorufin (1000 n*M*) or methoxyresorufin (500 n*M*) in the presence of NADPH and the amount of resorufin formed was determined by HPLC (Figs. 1 and 2). The resorufin

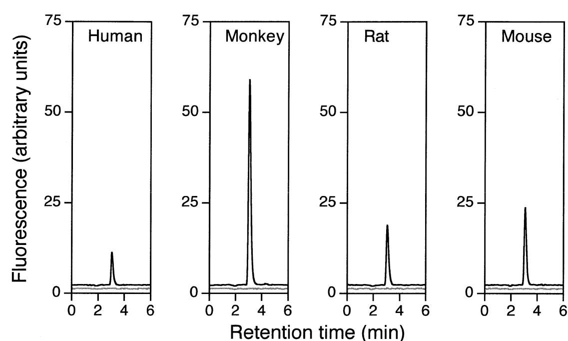

Fig. 1. HPLC analysis of EROD activity in liver microsomes from human, monkey, rat and mouse. Reactions were performed in the presence of ethoxyresorufin (1000 n*M*), NADPH (1 m*M*) and liver microsomal proteins (60 μg) in a total volume of 500 μl for 6 min at 25°C. The method of sample preparation and the HPLC conditions are described in Experimental. The gray lines indicate results for blank samples.

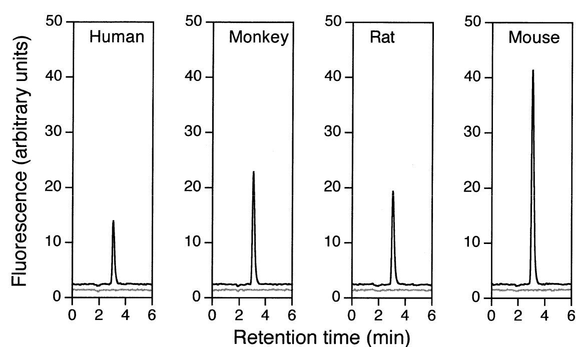

Fig. 2. HPLC analysis of MROD activity in liver microsomes from human, monkey, rat and mouse. Reactions were performed in the presence of methoxyresorufin (500 n*M*), NADPH (1 m*M*) and liver microsomal proteins (60 μg) in a total volume of 500 μl for 6 min at 25°C. The method of sample preparation and the HPLC conditions are described in Experimental. The gray lines indicate results for blank samples.

was eluted in 3.1 min, and no interfering peaks were detected under the conditions described. Ethoxyresorufin and methoxyresorufin do not themselves exhibit fluorescence intensities on HPLC, although ethoxyresorufin and methoxyresorufin were eluted in 5.9 and 4.6 min with UV detection, respectively.

3.2. Calibration curves and detection limit

Calibration curves ($y=ax+b$) were prepared from solution to which resorufin had been added in the range of 1.0–1000 pmol. A high correlation was found between the amount of resorufin (x: pmol/assay) and the peak area ratio (y: arbitrary units) ($n=6$, $y=0.999x+0.098$, $r=1.000$). The detection limit for resorufin (signal-to-noise ratio of 3) was 0.80 pmol/assay. The value on the column was 0.04 pmol, practically in accordance with a previous result obtained using the HPLC method [10]. The recoveries determined from liver microsomes of each animal species spiked with 50 pmol resorufin ($n=6$) were 98.6±2.2% for human, 98.2±2.2% for monkey, 98.8±1.7% for rat and 99.7±1.6% for mouse. The decomposition of resorufin was observed in a preliminary study using 50 μl of 10% (w/v) trichloroacetic acid or 50 μl of 60% (w/v) perchloric acid to stop the enzyme reactions. When the enzyme reactions were stopped with 50 μl of 20% (w/v) zinc sulfate, the recoveries of resorufin were only 65–71% (data not shown).

3.3. Accuracy and precision

The accuracy and precision of the method were determined by analysis of EROD and MROD activities of microsomal proteins from human, monkey, rat and mouse liver samples. Three samples were analyzed six times for the intra-day precision and three times daily for 6 days for the inter-day precision. The results are summarized in Table 1.

Table 1
Precision of the assay for EROD and MROD activities in liver microsomes from human, monkey, rat and mouse[a]

	EROD			MROD		
	n	Activity (mean±SD) (pmol/min/mg protein)	RSD (%)	*n*	Activity (mean±SD) (pmol/min/mg protein)	RSD (%)
Intra-day						
Human	6	25.5±0.7	2.7	6	30.2±1.0	3.3
Monkey	6	151±5	3.3	6	57.3±3.0	5.2
Rat	6	47.7±2.8	5.9	6	48.9±1.7	3.5
Mouse	6	58.6±2.6	4.4	6	104±5	4.8
Inter-day						
Human	6	24.9±0.8	3.2	6	32.2±1.5	4.7
Monkey	6	156±6	3.8	6	56.8±1.8	3.2
Rat	6	45.8±1.9	4.1	6	47.1±1.6	3.4
Mouse	6	59.3±1.7	2.9	6	108±2	1.9

[a] Reactions were performed in the presence of ethoxyresorufin (1000 n*M*) or methoxyresorufin (500 n*M*), NADPH (1 m*M*) and liver microsomal proteins (60 μg) in a total volume of 500 μl for 6 min at 25°C. The method of sample preparation and the HPLC conditions are described in Experimental.

Intra-day and inter-day precisions (expressed as relative standard deviation, RSD) were less than 6% for both enzyme activities. Accuracies ranged from 91.8 to 105% for EROD activity and 93.9 to 105% for MROD activity. Furthermore, the data were similar for the present method and the spectrofluorimetric method. The EROD and MROD activities in liver microsomes as determined by the spectrofluorimetric method [8] (n=6) were: 26.2±0.6 and 31.5±1.0 pmol/min/mg protein for human; 164±10 and 60.8±3.0 pmol/min/mg protein for monkey; 45.4±2.4 and 51.7±1.2 pmol/min/mg protein for rat; and 60.3±4.4 and 109±4 pmol/min/mg protein for mouse, respectively.

3.4. Linearity of resorufin formation

To determine the optimal reaction conditions for the assay of EROD and MROD activities in liver microsomes, the dependence of the enzyme activities on incubation time and microsomal protein amount was studied. For these studies, substrate concentrations of 1000 nM for EROD and 500 nM for MROD were used. When the determination for EROD and MROD activities in liver microsomes was performed at 37°C (60 μg of microsomal protein), the linearity of resorufin formation was 2–3 min in all animal species (data not shown). By contrast, the resorufin formation was linear from 6 to 10 min at 25°C in liver microsomes from all animal species (Fig. 3). Similarly, in both assays of EROD and MROD activities in liver microsomes (25°C), the resorufin formation was found to be linear up to 80–150 μg microsomal protein in all animal species (Fig. 4). Therefore, to reduce the experimental error (higher variation), 6 min of incubation time, 60 μg of microsomal protein and 25°C were used as the standard conditions for assays for EROD and MROD activities in this study.

3.5. Kinetic analysis

To compare the enzymology of EROD and MROD in liver microsomes from human, monkey, rat and mouse, Eadie–Hofstee plots were constructed. The curves and Michaelis–Menten parameters for the enzymes are shown in Fig. 5 and Table 2, respectively. EROD activities in human and monkey

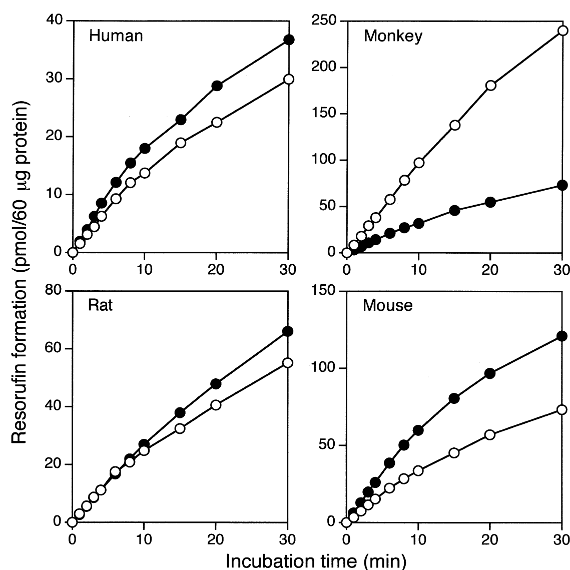

Fig. 3. Dependence on incubation time of EROD and MROD activities in liver microsomes from human, monkey, rat and mouse. Reactions were performed in the presence of ethoxyresorufin (1000 nM) or methoxyresorufin (500 nM), NADPH (1 mM) and liver microsomal proteins (60 μg) in a total volume of 500 μl for 0–30 min at 25°C. All assays were performed in triplicate. The method of sample preparation and the HPLC conditions are described in Experimental. Symbols are: ○, EROD; and ●, MROD. Each point represents the mean of two separate experiments. Similar results were obtained in both experiments.

liver microsomes indicated a monophasic kinetic pattern, whereas the pattern in rat and mouse liver microsomes was biphasic. K_{m1} for EROD activity was higher in human liver microsomes than in monkey, rat and mouse liver microsomes. V_{max1} for EROD activity was highest in monkey among the four animal species. No remarkable differences were found in K_{m2} and V_{max2} for EROD activity between rat and mouse liver microsomes. These results may mean that EROD activities in liver microsomes from rodents were catalyzed by at least two distinct P450 isoforms. On the other hand, the kinetics for MROD activities were monophasic in liver microsomes from all animal species. The values of K_m for MROD activities in human and monkey liver microsomes were higher than those of rat and mouse liver microsomes. In order of V_{max} for MROD activity in liver microsomes, the species ranked mouse>monkey>rat>human. Thus, the kinetics pattern for EROD and MROD activities in liver microsomes

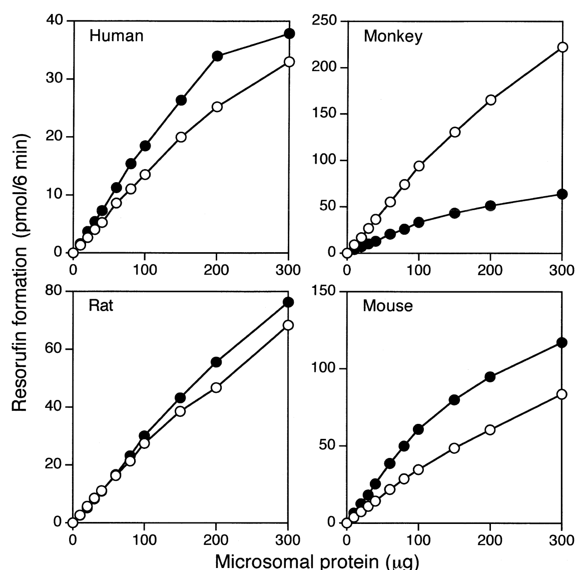

Fig. 4. Dependence on protein amount of EROD and MROD activities in liver microsomes from human, monkey, rat and mouse. Reactions were performed in the presence of ethoxyresorufin (1000 n*M*) or methoxyresorufin (500 n*M*), NADPH (1 m*M*) and liver microsomal proteins (0–300 μg) in a total volume of 500 μl for 6 min at 25°C. All assays were performed in triplicate. The method of sample preparation and the HPLC conditions are described in Experimental. Symbols are: ○, EROD; and ●, MROD. Each point represents the mean of two separate experiments. Similar results were obtained in both experiments.

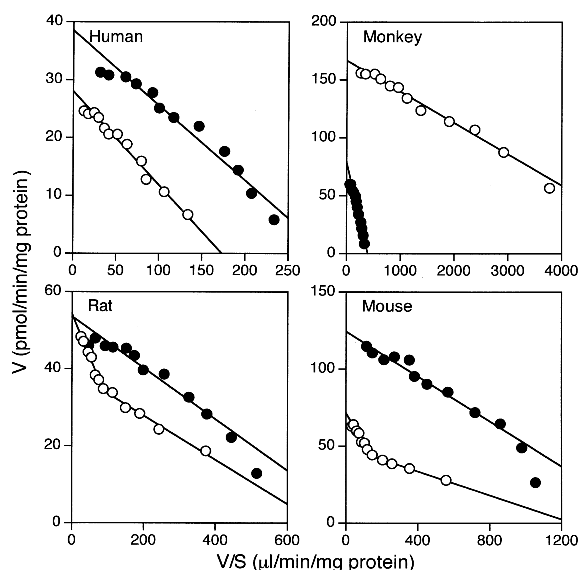

Fig. 5. Eadie–Hofstee plots of EROD and MROD activities in liver microsomes from human, monkey, rat and mouse. Reactions were performed in the presence of ethoxyresorufin (15–2000 n*M*) or methoxyresorufin (25–1000 n*M*), NADPH (1 m*M*) and liver microsomal proteins (15–60 μg) in a total volume of 500 μl for 6 min at 25°C. All assays were performed in triplicate. The method of sample preparation and the HPLC conditions are described in Experimental. Symbols are: ○, EROD; and ●, MROD. Each point represents the mean of two separate experiments. Similar results were obtained in both experiments.

differed extensively among the animal species. This species difference may depend on the ratio or function of *CYP1A* and other constitutive P450 isoforms in each animal species.

3.6. Induction study

The dependence on incubation time and protein amount of EROD and MROD activities in 3-methylcholanthrene-pretreated liver microsomes from rat and mouse was determined preliminarily at 25°C. The optimal reaction conditions for the determination of EROD and MROD activities in induced liver microsomes were within 20 μg of microsomal protein and within 6 min of incubation time in both animal species. Table 3 shows the activities of EROD and MROD in control and 3-methylcholanthrene-pretreated liver microsomes from rat and mouse. EROD activities in rat and mouse liver microsomes were highly induced by 3-methylcholanthrene, the levels relative to control activities increased 84- and 39-fold, respectively. 3-Methylcholanthrene also significantly induced MROD activities in liver microsomes from both animal species, although the induction was less potent than that for EROD. These induction profiles were similar to the results in a previous report [6].

4. Conclusion

EROD and MROD activities in mammalian liver microsomes have been reported to be specifically catalyzed by CYP1A isoforms [4–7]. Furthermore, as CYP1A isoforms in mammalian tissues are re-

Table 2
Michaelis–Menten parameters for EROD and MROD activities in liver microsomes from human, monkey, rat and mouse[a]

	EROD				MROD	
	K_{m1} (nM)	V_{max1} (pmol/min/mg protein)	K_{m2} (nM)	V_{max2} (pmol/min/mg protein)	K_m (nM)	V_{max} (pmol/min/mg protein)
Human	162	28.0			130	38.5
Monkey	27.0	167			199	78.8
Rat	57.4	39.3	227	54.2	66.7	53.6
Mouse	38.8	49.1	190	71.3	73.0	125

[a] Reactions were performed in the presence of ethoxyresorufin (15–2000 nM) or methoxyresorufin (25–1000 nM), NADPH (1 mM) and liver microsomal proteins (15–60 μg) in a total volume of 500 μl for 6 min at 25°C. All assays were performed in triplicate. The method of sample preparation and the HPLC conditions are described in Experimental. Each value represents the mean of two separate experiments and was obtained from Eadie–Hofstee plots. Similar results were obtained in both experiments.

Table 3
Induction of EROD and MROD activities by 3-methylcholanthrene in liver microsomes from rat and mouse[a]

	EROD (pmol/min/mg protein)		MROD (pmol/min/mg protein)	
	Control	3-Methylcholanthrene	Control	3-Methylcholanthrene
Rat	42.8±3.7	3593±342*	39.4±4.3	1124±105*
Mouse	57.3±2.9	2219±177*	102±12	1936±155*

[a] Reactions were performed in the presence of ethoxyresorufin (1000 nM) or methoxyresorufin (500 nM), NADPH (1 mM) and liver microsomal proteins (15 μg) in a total volume of 500 μl for 6 min at 25°C. All assays were performed in triplicates. The method of sample preparation and the HPLC conditions are described in Experimental. Each value represents the mean±SD of three individual animals.

*Significantly different from control by Student's t-test ($P<0.0001$).

markably induced by some xenobiotic chemicals, EROD and MROD activities have been also used as toxicological markers for environmental chemicals [1,3]. However, the expression levels of CYP1A isoforms are very low in normal animals [5,6], and high sensitivity is needed to determine CYP1A activities from biological samples. Furthermore, no method for the determination of non-induced and highly induced activities of EROD and MROD under the same assay conditions has been developed because of their selective and remarkable induction. With the method described here, the kinetics and inducibility of EROD and MROD activities in mammalian liver were precisely determined using small amounts of microsomal proteins without extraction. Therefore, this method for the determination of EROD and MROD activities is sensitive and efficient, and should be useful for in vivo and in vitro studies on the interaction of xenobiotic chemicals with CYP1A isoforms in mammals.

Acknowledgements

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