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Highly sensitive high-performance liquid chromatographic assay for coumarin 7-hydroxylation and 7-ethoxycoumarin *O*-deethylation by human liver cytochrome P450 enzymes

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Abstract

A highly sensitive method for the determination of coumarin 7-hydroxylation and 7-ethoxycoumarin *O*-deethylation by human cytochrome P450 (P450 or CYP) enzymes was developed using high-performance liquid chromatography (HPLC). The newly developed HPLC method was found to be about 100-fold more sensitive than the previous spectrofluorimetric method in detecting the metabolite 7-hydroxycoumarin (umbelliferone). With this high sensitivity, the kinetics of coumarin 7-hydroxylation and 7-ethoxycoumarin *O*-deethylation catalyzed by human liver microsomal and recombinant P450 enzymes were determined more precisely. With 36 different substrate concentrations in these two reactions, coumarin 7-hydroxylation was found to be catalyzed mainly by a single enzyme CYP2A6 and 7-ethoxycoumarin was oxidized by at least two enzymes CYP2E1 and CYP1A2 in human liver microsomes.

Keywords: Enzymes; Coumarin; Cytochromes

1. Introduction

Cytochrome P450 (P450 or CYP) comprises a superfamily of enzymes that catalyze oxidation of numerous xenobiotic chemicals such as drugs, toxic chemicals, and carcinogens as well as endobiotic chemicals such as steroids, fatty acids, prostaglandins, and vitamins [1,2]. Most of the xenobiotic chemicals have been shown to be catalyzed mainly by one to three families of P450s and individual P450 enzymes have considerable, but overlapping, substrate specificities [2,3].

Coumarin has been shown to be a marker substrate for CYP2A6, while 7-ethoxycoumarin has been shown to be metabolized by several P450 enzymes in human liver microsomes [4,5]. We have previously shown using a spectrofluorimetric method that 7-ethoxycoumarin *O*-deethylation is catalyzed by several P450 enzymes including CYP1A2 and CYP2E1 in human liver microsomes [6]. Recent studies have revealed that two or more P450 enzymes are sometimes able to oxidize a single substrate where one enzyme has high affinity and the other(s) has (have) low affinities for the substrate [7,8]. One of the suggested procedures to define which P450 enzymes are more active in the reactions is to determine kinetically the activities with a wide range of substrate concentrations [8]. In order to determine more precisely the roles of several human P450 enzymes

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in the metabolism of coumarin and 7-ethoxycoumarin, it is of great value to develop a highly sensitive HPLC method for the determinations of 7-hydroxycoumarin (umbelliferone) which is a product formed through coumarin 7-hydroxylation and 7-ethoxycoumarin *O*-deethylation by P450 enzymes [4,6].

In this study, we report a simple and sensitive method for the determination of coumarin 7-hydroxylation and 7-ethoxycoumarin *O*-deethylation by human P450 enzymes using HPLC; both reactions have been shown to give the same product 7-hydroxycoumarin [9]. Liver microsomes from different human samples or recombinant human P450 enzymes in human lymphoblastoid cell and baculovirus systems [(all were purchased from Gentest (Woburn, MA, USA)] were used as enzyme sources. Kinetic analysis for the coumarin 7-hydroxylation and 7-ethoxycoumarin *O*-deethylation by human P450 enzymes is reported using 36 different concentrations of these two substrates.

2. Experimental

2.1. Chemicals

Coumarin and 7-ethoxycoumarin were obtained from Sigma (St Louis, MO, USA), and 7-hydroxycoumarin from Katayama (Osaka, Japan). Other drug substrates, their metabolites, and reagents used in this study were obtained from sources as described previously or of highest qualities commercially available [10].

2.2. Enzyme preparation

Human liver samples were obtained from organ donors or patients undergoing liver resection as described previously [10,11]. Liver microsomes were prepared as described and suspended in 10 m*M* Tris–HCl buffer (pH 7.4) containing 1.0 m*M* EDTA and 20% glycerol (v/v) [12]. Human recombinant CYP1A2, 1B1, 2A6, 2B6, 2E1, and 3A4 in microsomes of human lymphoblast cells, co-expressing human NADPH-P450 reductase were purchased from Gentest. Other recombinant P450 enzymes including CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5, and 4A11 in a baculovirus system co-expressing human NADPH-P450 reductase were also obtained from Gentest; the P450 contents in these systems were used as described in the data sheets provided by the manufacturer.

P450 was estimated spectrally by the method of Omura and Sato [13]. Protein concentrations were estimated by the method of Lowry et al. [14].

2.3. Incubations

Coumarin 7-hydroxylation and 7-ethoxycoumarin *O*-deethylation activities by P450 enzymes were determined by the previous method using a spectrofluorimeter [6,12,15] or the present method using HPLC. In both cases, incubation mixtures consisted of human liver microsomes (0.025 mg protein/ml) or recombinant P450 (10 pmol/ml) with several concentrations of coumarin or 7-ethoxycoumarin in a final volume of 0.20 ml of 100 m*M* potassium phosphate buffer (pH 7.4) containing a NADPH-generating system (0.5 m*M* $NADP^+$, 5 m*M* glucose-6-phosphate, and 0.5 unit of glucose-6-phosphate dehydrogenase/ml). Incubations were carried out at 37°C for 10 min and terminated by adding 10 μl of 60% $HClO_4$ (w/v) for the HPLC method or 10 μl of 10% Cl_3CCO_2H (w/v) for the spectrofluorimetric method.

2.4. HPLC system

After terminating the reaction by adding 10 μl of 60% $HClO_4$ (w/v), the denatured protein was removed by centrifugation at 900 *g* for 5 min and the aliquot (usually 25 μl) of the supernatant was used for HPLC analysis with a LC-CCPS system (Tosoh, Tokyo, Japan) with a spectrofluorimeter FS-8020 (Tosoh). Separation was done with a C_{18} 5-μm analytical column (Mightsil RP-18, 150×4.6 mm I.D., Kanto, Tokyo, Japan) equipped with a C_{18} 5-μm guard column (Mightsil RP-18, 5-4.6 mm, Kanto). The eluent consisted of a mixture of 45% CH_3CN (v/v) containing 20 m*M* $NaClO_4$ (pH 2.5). The flow-rate was 1.2 ml/min and the fluorimetric detection was done at an excitation wavelength of 338 nm and an emission wavelength of 458 nm. Peak areas thus obtained were integrated with a

Chromatopac Instrument (C-R6A Chromatopac, Shimadzu, Kyoto, Japan).

2.5. Spectrofluorimetric system

After terminating the reaction with 10 μl of 10% Cl_3CCO_2H (w/v), the metabolites were extracted with 1.0 ml of CH_2Cl_2. The mixtures were centrifuged at 900 *g* for 5 min and the aliquots (usually 0.5 ml) of the organic layer (lower layer) were re-extracted with 3.0 ml of 30 m*M* sodium borate (pH 9.0). The formation of 7-hydroxycoumarin was determined fluorimetrically at the excitation wavelength of 338 nm and the emission wavelength of 458 nm with a Shimadzu RF-5000 spectrofluorimeter (Shimadzu, Kyoto).

2.6. Kinetic analysis

Kinetic parameters for coumarin 7-hydroxylation and 7-ethoxycoumarin *O*-deethylation by human P450 enzymes were estimated using a computer program (KaleidaGraph program from Synergy Software, Reading, PA, USA) designed for nonlinear regression analysis of a hyperbolic Michaelis–Menten equation.

3. Results

3.1. Comparison of sensitivity of spectrofluorimetric and HPLC systems for the detection of 7-hydroxycoumarin

Different concentrations of 7-hydroxycoumarin were determined with respect to the fluorescence intensities with spectrofluorimetric and HPLC methods (Fig. 1). Fluorescence intensities increased linearly between 0.05 and 10 μ*M* 7-hydroxycoumarin in the spectrofluorimetric method and between 0.0005 and 10 μ*M* in the HPLC method.

3.2. Coumarin 7-hydroxylation and 7-ethoxycoumarin O-deethylation by human P450 enzymes

Liver microsomes from human sample HL-18 were incubated with coumarin or 7-ethoxycoumarin in the presence of an NADPH-generating system and the 7-hydroxycoumarin formed was determined using HPLC (Fig. 2). Coumarin itself does not have fluorescence intensities and only the product 7-hydroxycoumarin was detected just after the peak of NADPH on HPLC analysis. In both coumarin 7-hydroxylation and 7-ethoxycoumarin *O*-deethyla-

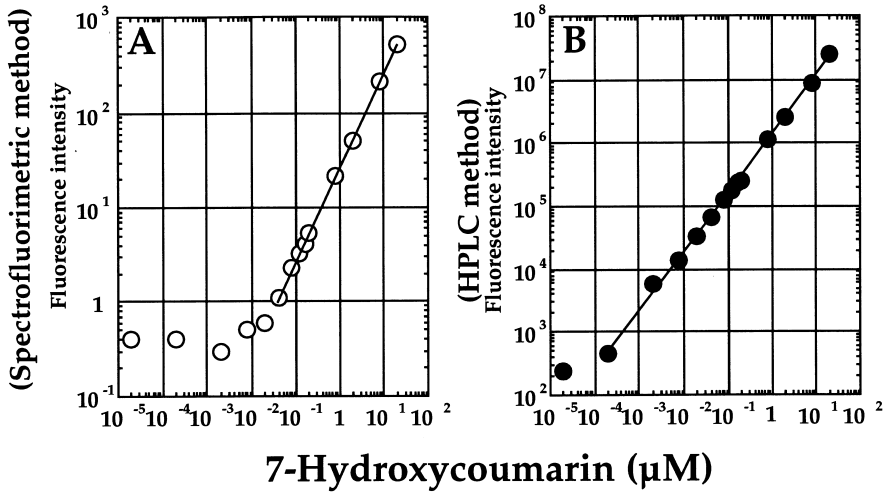

Fig. 1. Determination of 7-hydroxycoumarin at different concentrations by spectrofluorimetric (A) and HPLC (B) methods. 7-Hydroxycoumarin was dissolved in methanol and then diluted with 100 m*M* potassium phosphate buffer to make desired concentrations and the fluorescence intensities were detected with an excitation wavelength at 338 nm and emission wavelength at 458 in both methods. Each point was from a single determination; the experiments were repeated two times and the results obtained were essentially similar in these two cases.

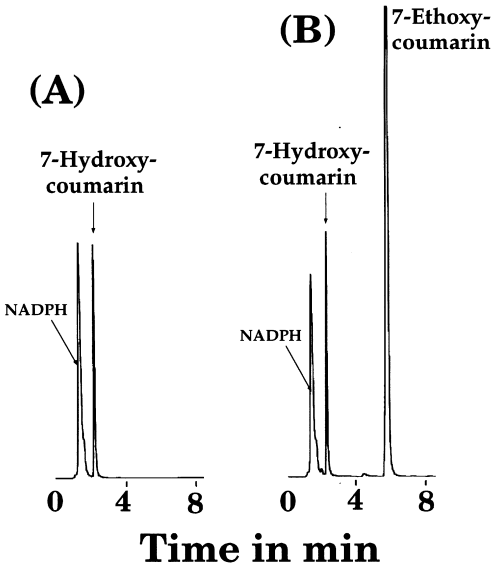

Fig. 2. HPLC analysis of the coumarin 7-hydroxylation (A) and 7-ethoxycoumarin *O*-deethylation (B) by liver microsomes of human sample HL-18.

tion reaction by human liver microsomes, product formation on HPLC analysis was found to be linear up to 0.02 mg and 0.03 mg protein, respectively (Fig. 3).

Activities of coumarin 7-hydroxylation and 7-ethoxycoumarin *O*-deethylation by liver microsomes of 24 human samples were determined and compared in spectrofluorimetric and HPLC methods (Fig. 4). There were good correlations between these two

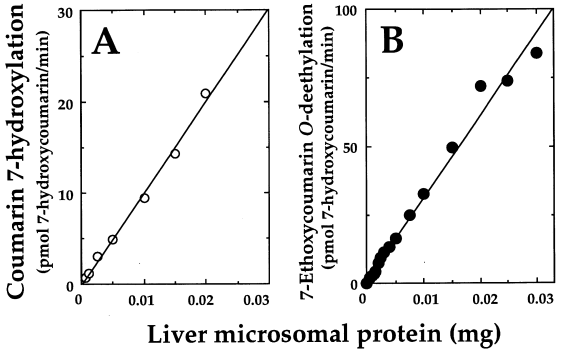

Fig. 3. Dependence on protein concentrations of the coumarin 7-hydroxylation (A) and 7-ethoxycoumarin *O*-deethylation (B) by liver microsomes of human sample HL-18.

methods in both reactions catalyzed by human liver microsomes.

3.3. Kinetic analysis of coumarin 7-hydroxylation and 7-ethoxycoumarin O-deethylation by human P450 enzymes

Coumarin 7-hydroxylation by liver microsomes from human sample HL-18 was determined with the HPLC method measuring 36 different concentrations of coumarin between 0.17 μ*M* and 100 μ*M* (Fig. 5). Eadie-Hofstee plot (Fig. 5B) indicated the association of a single P450 enzyme in the coumarin 7-hydroxylation reaction (K_m, 0.76 μ*M*; V_{max}, 0.39 nmol/min/mg protein). 7-Ethoxycoumarin *O*-deethylation wwas also determined in liver microsomes of HL-18 at 36 different substrate concentrations between 7.5 μ*M* and 1 m*M* (Fig. 6). Eadie-Hofstee plot (Fig. 6B) demonstrated that there were at least two components in 7-ethoxycoumarin *O*-deethylation by human liver microsomes. One component was determined to be an enzyme having a K_m value of 16 μ*M* and a V_{max} value of 0.17 nmol/min/mg protein. The other was to have a K_m value of 150 μ*M* and a V_{max} value of 0.46 nmol/min/mg protein.

Recombinant human CYP1A2, 1B1, 2A6, 2B6, 2E1, and 3A4 enzymes in microsomes of human lymphoblast cells, co-expressing human NADPH-P450 reductase, were used to determine which P450 enzymes are more important in catalyzing coumarin 7-hydroxylation and 7-ethoxycoumarin *O*-deethylation (Table 1). Of six recombinant P450 enzymes examined, only CYP2A6 was highly active in catalyzing coumarin 7-hydroxylation with a K_m value of 0.4 μ*M* and a V_{max} value of 0.56 nmol/min/nmol P450. There were several P450 forms which are active in catalyzing 7-ethoxycoumarin *O*-deethylation reaction in recombinant systems. Similar activities were determined in CYP1A2 and 1B1 in which V_{max} values determined were between 0.1 and 0.2 nmol/min/nmol P450 with K_m values of ~30 μ*M*. CYP2E1 had the highest V_{max} value of 1.8 nmol/min/nmol P450 with a K_m value of 109 μ*M*. CYP2B6 gave a turnover number of 0.82 nmol/min/nmol P450 with a K_m value of 190 μ*M*. CYP3A4 had very high K_m value of ~1 m*M*.

We also determined the coumarin 7-hydroxylation

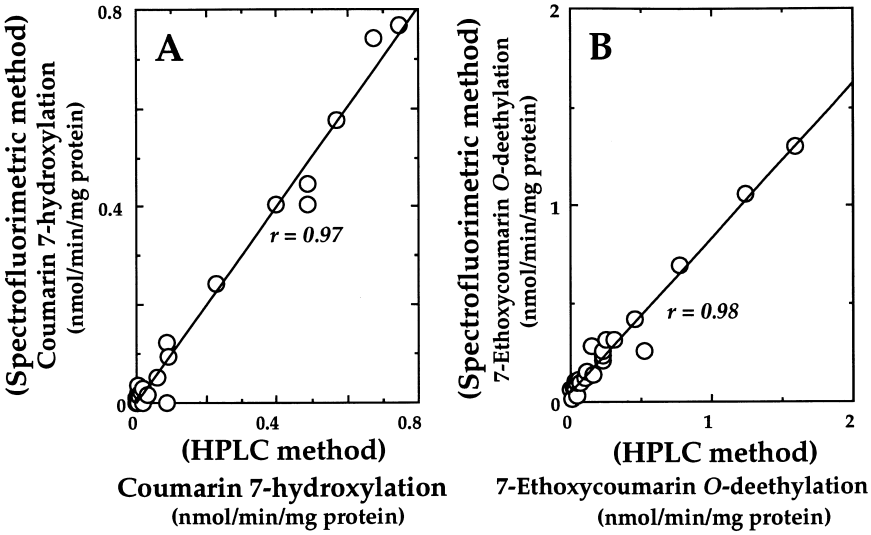

Fig. 4. Comparison of activities of coumarin 7-hydroxylation (A) and 7-ethoxycoumarin *O*-deethylation (B) by liver microsomes from 24 different human samples in spectrofluorimetric and HPLC methods.

activities by 14 forms of recombinant human P450 enzymes in baculovirus systems which co-express human NADPH-P450 reductase (Fig. 7). The P450 enzymes used were CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5, and 4A11 and substrate concentrations employed were 1 μ*M*, 10 μ*M*, and 100 μ*M*. Of these P450 enzymes determined at three substrate concentrations, only CYP2A6 was found to be highly active in catalyzing coumarin 7-hydroxylation activities.

4. Discussion

HPLC analysis for the detection of 7-hydroxycoumarin was found to be of great use for the assay

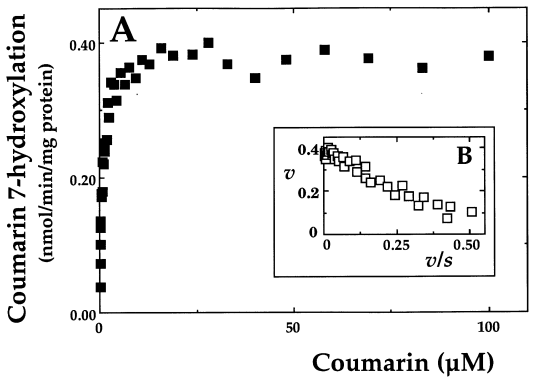

Fig. 5. Dependence on substrate concentration of coumarin 7-hydroxylation by liver microsomes of human sample HL-18 (A). Insert (B) shows the kinetic analysis of the reaction.

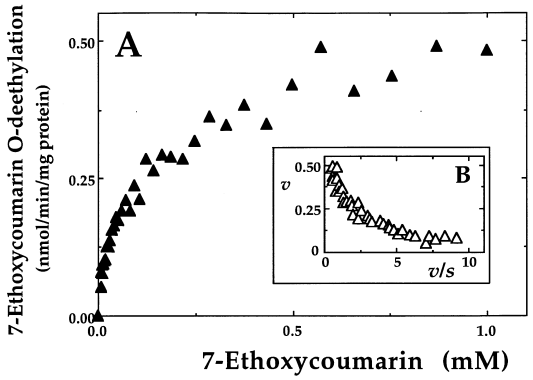

Fig. 6. Dependence on substrate concentration of 7-ethoxycoumarin *O*-deethylation by liver microsomes of human sample HL-18 (A). Insert (B) shows the kinetic analysis of the reaction.

Table 1
Kinetic analysis of coumarin 7-hydroxylation and 7-ethoxycoumarin *O*-deethylation by human P450 enzymes expressed in human lymphoblast cell lines[a]

P450	Coumarin 7-hydroxylation		7-Ethoxycoumarin *O*-deethylation	
	K_m (μM)	V_{max} (nmol/min/nmol P450)	K_m (μM)	V_{max} (nmol/min/nmol P450)
CYP1A2		<0.005	31±7	0.17±0.01
CYP1B1		<0.005	27±6	0.13±0.01
CYP2A6	0.4±0.1	0.56±0.04		<0.05
CYP2B6		<0.005	190±49	0.82±0.10
CYP2E1		<0.005	109±23	1.80±0.18
CYP3A4		<0.005	980±85	0.95±0.31

[a] Kinetic analysis was determined by the method as described in section 2. Data are means and S.E.

of coumarin 7-hydroxylation and 7-ethoxycoumarin *O*-deethylation by human P450 enzymes. After the termination of reactions, 7-hydroxycoumarin was directly separated in HPLC without extraction from the incubation mixture. The sensitivity of HPLC method was found to be about 100-fold higher than those of a previous spectrofluorimetric method for the detection of 7-hydroxycoumarin [4]. We also found that there were good correlations between these two methods for the determinations of coumarin 7-hydroxylation and 7-ethoxycoumarin *O*-deethylation activities catalyzed by liver microsomes of 24 human samples.

Using this newly developed HPLC method, the kinetics of coumarin 7-hydroxylation and 7-ethoxycoumarin *O*-deethylation catalyzed by human P450 enzymes were determined very precisely. The substrate concentrations used for the former reaction were between 0.17 μM and 100 μM and those for the latter reaction were between 7.5 μM and 1 m*M*. As has been reported previously, coumarin 7-hydroxylation was found to be catalyzed by a single enzyme CYP2A6 in purified P450 enzymes and human liver microsomes [4,5]. In good agreement with this previous view, we found in this study that of 14 forms of recombinant human P450 enzymes examined in the baculovirus systems, only CYP2A6 was highly active in catalyzing coumarin 7-hydroxylation activities at substrate concentrations of 1, 10, and 100 μM (Fig. 7). The K_m value of 0.4 μM determined with recombinant CYP2A6 in human lymphoblast cells was very similar to that (0.76 μM) determined by liver microsomes of human sample HL-18 (Table 1 and Fig. 5).

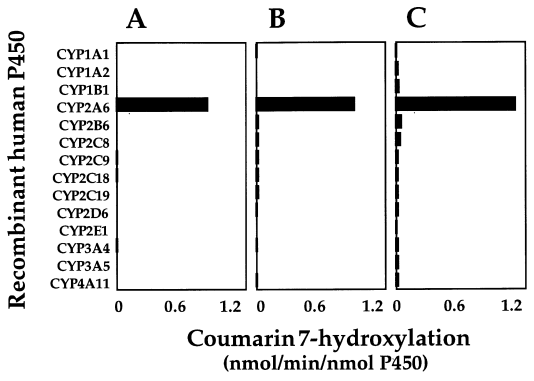

Fig. 7. Coumarin 7-hydroxylation activities by recombinant human P450 enzymes expressed in a baculovirus system. Substrate concentrations used were 1 μM (A), 10 μM (B), and 100 μM (C). Data are means of duplicate determinations.

Previously, we have reported that 7-ethoxycoumarin *O*-deethylation is catalyzed by at least two P450 enzymes in human liver microsomes; the conclusion reached from the results of inhibition of catalytic activities by anti-P450 antibodies in different human samples [6]. CYP2E1 is shown to be a high K_m and high V_{max} enzyme and CYP1A2 is a low K_m and low V_{max} enzyme, and the present results supported these results using 36 different substrate concentrations for the analysis of 7-ethoxycoumarin *O*-deethylation by human P450 enzymes. CYP1B1 and 2B6 were also found to have substantial activities for 7-ethoxycoumarin *O*-deethylation. However, it should be mentioned that these two P450

forms are shown to be minor components in human liver microsomes [10,16].

In conclusion, a newly developed HPLC method for the determination of coumarin 7-hydroxylation and 7-ethoxycoumarin *O*-deethylation activities is suggested to be of great use for the characterization of the roles of P450 enzymes in humans. This method is also applicable to the determination of enzymes which have low catalytic activities, present in several organisms such as plants and vegetables.

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