RECOMBINANT HUMAN CYP1A2 CATALYSES THE O-DEMETHYLATION OF 7-METHOXYCOUMARIN

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Abstract

1. HPLC conditions were established for the separation of 7-ethoxycoumarin, 7-methoxycoumarin and 7-hydroxycoumarin (umbelliferone).

2. The 7-methoxycoumarin, purchased from a commercial source, was cleaned-up by solid-phase extraction to remove a small amount (0.027%) of contaminating umbelliferone.

3. In a pilot experiment, recombinant human CYP1A2 was shown to catalyse 7-methoxycoumarin O-demethylase (MCOD) activity.

4. Incubation conditions were optimised to give linear formation of product with respect to incubation time and CYP concentration, at a substrate concentration < K_m. In CYP1A2R Bactosomes, the reaction was linear for 15 min, and up to a CYP1A2 concentration of 5 pmol/ml, at 2 µM 7-methoxycoumarin

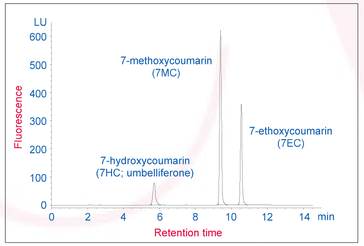
5. MCOD activity in CYP1A2R Bactosomes was characterised by a **V_{max} of 3.0 ± 0.9 nmol/min/nmol CYP**, and a **K_m of 4.1 ± 0.5 µM** (mean ± SD; n=3). Corresponding kinetic parameters for 7-ethoxycoumarin O-deethylase (ECOD) activity were V_{max} 0.6 ± 0.1 nmol/min/nmol CYP and K_m 1.5 ± 0.2 µM.

Introduction and Aim

The O-deethylation of 7-ethoxycoumarin (7EC) is catalysed by multiple recombinant human CYPs (1). In contrast, very little is known about the role of human CYPs in the O-dealkylation of other 7-alkoxy-coumarins. This is perhaps somewhat surprising, given the wealth of such comparative data relating to the O-dealkylation of the 7-alkoxyresorufins.

The aim of this study was therefore to establish an assay for the determination of 7-methoxycoumarin O-demethylase (MCOD) activity, and subsequently to investigate a possible role for human CYP1A2 in catalysing this reaction.

Figure 1: Chromatogram showing separation of 7HC, 7MC and 7EC



A sample consisting of 7HC (5 ng/ml), 7MC (3.52 µg/ml) and 7EC (1.90 µg/ml) in 50 mM potassium phosphate buffer, pH 7.4 (1 ml) was prepared, then 125 µl of 1 M hydrochloric acid was added. Following centrifugation, 50 µl of supernatant was injected onto the HPLC, using the conditions as described in Materials and Methods.

Materials and Methods

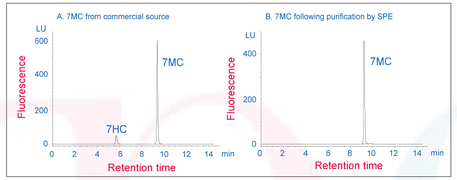
The chemicals 7-ethoxycoumarin and umbelliferone (7HC) were purchased from Sigma, and 7-methoxycoumarin (7MC; 98+%) came from Aldrich. HPLC grade acetonitrile and methanol were from Fisher. All other chemicals were supplied by BDH.

HPLC analysis was carried out using an Agilent 1100 Series instrument. Separation of 7EC, 7MC and 7HC was achieved on a Hypersil ODS (5 µm) 250 x 4.6 mm column at 30ºC, using an initial mobile phase of 0.05% orthophosphoric acid/acetonitrile (75/25; v/v) and a linear gradient of acetonitrile (25 to 65% from 3 to 8 min), at a flow rate of 1 ml/min. Detection was by fluorescence, using excitation and emission wavelengths of 324 and 458 nm, respectively.

Clean-up of the commercially-sourced 7MC was carried out by solid-phase extraction using a Mega Bond Elut C18 cartridge (Varian). The 7MC (19.3 mg) was dissolved in 11 ml methanol to make a 10 mM stock solution, which was then diluted by the addition of 33 ml water. This was loaded onto a C18 cartridge which had been primed with methanol and then equilibrated in 25% methanol. After washing with 100 ml of 25% methanol, the 7MC was eluted with 25 ml methanol. The solvent was then evaporated to dryness and the residue resuspended in 2 ml methanol. Purified 7MC was quantified by HPLC.

Incubations for the determination of MCOD activity were carried out at 37ºC in 50 mM potassium phosphate buffer (pH 7.4) containing 5 mM magnesium chloride and a NADPH regenerating system [5 mM glucose 6-phosphate, 1 mM $NADP^+$, 1 U/ml glucose 6-phosphate dehydrogenase], in a total volume of 200 µl. Reactions were terminated by the addition of 20 µl of 1 M hydrochloric acid. Incubations were carried out on CYP1A2R Bactosomes (three separate batches) and Reductase Bactosomes. Kinetic parameters were estimated by fitting the experimental data directly to the Michaelis-Menten equation using Microsoft Excel

Figure 2: Chromatograms showing clean-up of 7MC by solid-phase extraction



Chromatograms showing the purity of 7MC before (A) and after (B) clean-up by solid-phase extraction. See Materials and Methods for details. Injection volume was 50 µl.

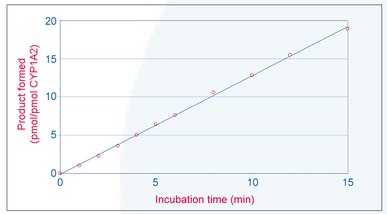
Results (1)

A reversed-phase HPLC gradient separation of 7HC, 7MC and 7EC was established (Figure 1). Retention times for the three compounds were 5.7 min (7HC), 9.4 min (7MC) and 10.5 min (7EC). The same HPLC conditions could also be used to analyse 4-methylumbelliferone (retention time 7.4 min; data not shown), allowing this compound to be used as an internal standard, if required. Limits of detection for 7HC and 7MC (taken as three times signal/noise ratio) were 0.47 and 101 pg/200 µl, respectively.

The sensitivity of the fluorometric method revealed a small amount of 7HC (0.027%), present as an impurity in the commercially-sourced 7MC (Figure 2A). This level of impurity was sufficient to interfere with the subsequent determination of MCOD activity.

Consequently, the 7MC was purified by solid-phase extraction (see Methods section for details). A total of 5.93 mg of purified 7MC was obtained, representing a recovery of 31%. With further optimisation, it is anticipated that the recovery of 7MC could be greatly improved. HPLC analysis showed that the purified 7MC was essentially free of contaminating 7HC (Figure 2A).

Figure 3: Linearity of product formation with respect to incubation time in CYP1A2R Bactosomes



Incubations were carried out as described in Materials and Methods, using a CYP1A2 concentration of 0.5 pmol/200 µl, and 2 µM 7MC.

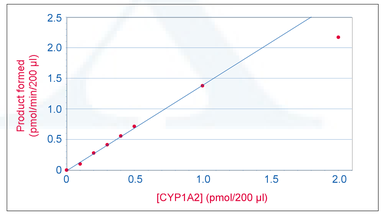
Results (2)

A pilot experiment was carried out to assess the ability of CYP1A2 to catalyse the O-demethylation of 7MC. MCOD activity in CYP1A2R Bactosomes was 4.13 nmol/min/nmol CYP at 20 µM 7MC, based on a 5 min incubation at a CYP concentration of 0.5 pmol/200 µl. There was no detectable conversion of 7MC to 7HC when Bactosomes containing CYP-reductase only were substitued for CYP1A2R Bactosomes in the incubation, thus ruling out the involvement of either the CYP-reductase alone or a bacterial membrane protein in the reaction.

Incubation conditions were then optimised to ensure linear formation of product with respect to incubation time (Figure 3) and CYP concentration (Figure 4), at a substrate concentration less than K_m. In CYP1A2R Bactosomes, the reaction was linear for 15 min (Fig. 3), and up to a CYP concentration of 5 pmol/ml (Fig. 4), at 2 µM 7MC.

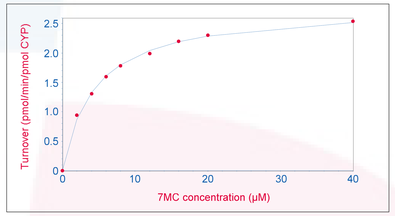
Using the optimised incubation conditions, Michaelis-Menten kinetic parameters were determined for MCOD activity in CYP1A2R Bactosomes (Figure 5). The reaction was characterised by a V_{max} of 3.0 ± 0.9 nmol/min/nmol CYP, and a K_m of 4.1 ± 0.5 µM (mean ± SD; n=3). Corresponding kinetic parameters for 7-ethoxycoumarin O-deethylase (ECOD) activity were V_{max} 0.6 ± 0.1 nmol/min/nmol CYP and K_m 1.5 ± 0.2 µM (mean ± SD; n=3). Intrinsic clearances for the two substrates (V_{max}/K_m) were 0.73 (7MC) and 0.40 (7EC) µl/min/pmol CYP.

Figure 4: Linearity of product formation with respect to CYP concentration in CYP1A2R Bactosomes



Incubations were carried out as described in Materials and Methods, using an incubation time of 5 min, and 2 µM 7MC.

Figure 5: Determination of Michaelis-Menten kinetic parameters for MCOD activity in CYP1A2R Bactosomes



Incubations were carried out as described in Materials and Methods, using an incubation time of 5 min, and a CYP1A2 concentration of 0.5 pmol/200 µl, at 2 µM 7MC.

Conclusions

1. 7HC, 7MC and 7EC can be separated by reversed-phase HPLC, using an acetonitrile gradient. Fluorometric detection allows the detection of very small (< 1 pg/200 µl) amounts of the 7HC metabolite.

2. Recombinant human CYP1A2 demonstrates MCOD activity. The intrinsic clearance of 7MC is almost double that of 7EC for the O-dealkylation reaction.

3. Investigation into the O-demethylation of 7MC by a panel of human CYPs could provide useful data, especially when compared with the corresponding data generated for 7EC (1).

Reference

1. Shimada, T, Tsumura, F and Yamazaki, H
"Prediction of human liver microsomal oxidations of 7-ethoxycoumarin and chlorzoxazone with kinetic parameters of recombinant cytochrome P-450 enzymes"
Drug Metab. Dispos. **27** (1999) : 1274-80

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