Enzymic Production of Drug Metabolites Using Recombinant Human Cytochrome P450s.

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Introduction

Despite being used routinely in *in vitro* metabolism studies, there is little information available on the use of recombinant cytochrome P450s to produce drug metabolites. Enzymatic synthesis represents a cost effective alternative to chemical synthesis and, where chemical synthesis proves ineffective may be the only means available to make a given metabolite. Here we demonstrate the efficient enzymatic production of diclofenac and paclitaxel metabolites using recombinant CYP2C9 and CYP2C8 respectively.

Methods

Diclofenac and glucose-6-phosphate dehydrogenase were obtained from Sigma Aldrich, UK. All other chemicals were from VWR International, UK.

Recombinant human cytochrome P450s were co-expressed with recombinant human NADPH P450 reductase in *E. coli* in 10 litre fermenters using standard procedures. Bacterial membranes were prepared from harvested bacteria by differential centrifugation following lysozyme treatment and sonication. Osmotically shocked bacteria were produced as follows; harvested *E. coli* were resuspended in 0.05 culture volumes hypertonic buffer (100 mM tris-acetate pH 7.6, 0.5 M sucrose, 0.5 mM EDTA). Once the bacterial pellet had been resuspended 0.05 culture volumes deionised water was added. HPLC analysis was carried out on an Agilent 1100 system using a Hypersil BDS C18 column (obtained from Crawford Scientific, UK).

Results

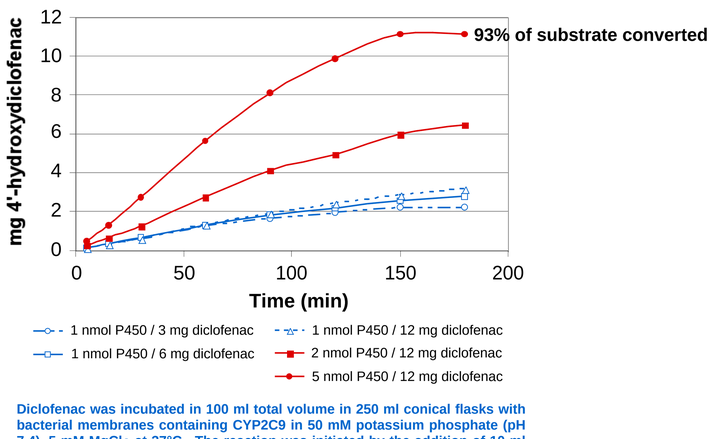
CYP2C9 / Diclofenac:

Incubation of diclofenac with bacterial membranes containing recombinant human CYP2C9 coexpressed with NADPH P450 reductase (CYP2C9HR Bactosomes) resulted in the efficient production of the metabolite 4'-hydroxydiclofenac (Fig. 1). HPLC analysis of the incubation mix (Fig. 3) demonstrated that the only detectable metabolite was 4'-hydroxydiclofenac demonstrating the specificity of the reaction. This was supported by the finding that all of the parent compound lost was accounted for in the metabolite produced.

Where the recombinant P450 is incorporated as part of a bacterial membrane preparation, exogenous co-factor, NADPH, must be added to the reaction (in this case, NADPH generating system was added) which adds significantly to the cost of carrying out the metabolism. In Fig. 2 we show that efficient conversion of diclofenac to its 4'-hydroxy metabolite can be achieved using osmotically shocked *E. coli* co-expressing CYP2C9 and NADPH P450 reductase. In this case, endogenous NADPH supplied by bacterial metabolism is used and no exogenous supply is required, significantly reducing the cost of carrying out the metabolism.

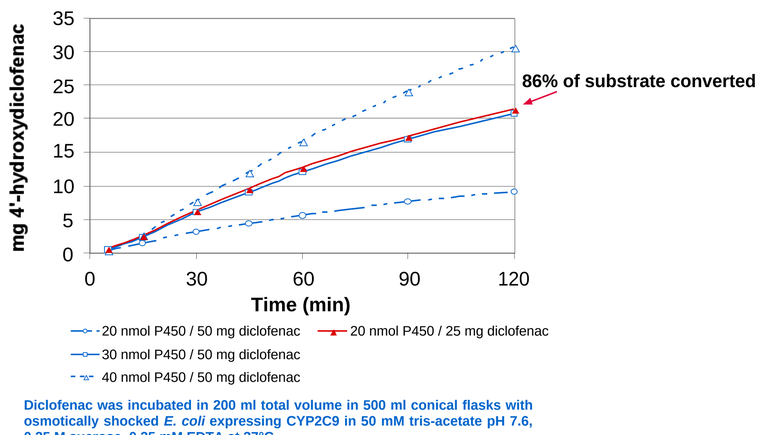
High concentrations of diclofenac were used (the lowest being 30 µg/ml or 94 µM which is 30 x the Km of the enzyme). There was no evidence of substrate inhibition occuring as altering the concentration of substrate had no significant effect on the rate of metabolite generation (blue lines Fig. 1).

Fig. 1. 4'-hydroxydiclofenac production with bacterial membranes containing CYP2C9



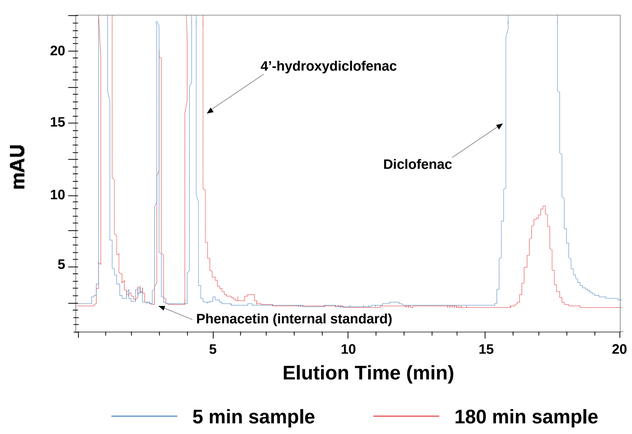
Diclofenac was incubated in 100 ml total volume in 250 ml conical flasks with bacterial membranes containing CYP2C9 in 50 mM potassium phosphate (pH 7.4), 5 mM $MgCl_2$ at 37°C. The reaction was initiated by the addition of 10 ml 50 mM potassium phosphate pH 7.4 containing 4.0 mg/ml glucose-6-phosphate, 4.2 mg/ml NADP and 35 units/ml glucose-6-phosphate dehydrogenase.

Fig. 2 4'-hydroxydiclofenac production with *E. coli* containing CYP2C9



Diclofenac was incubated in 200 ml total volume in 500 ml conical flasks with osmotically shocked *E. coli* expressing CYP2C9 in 50 mM tris-acetate pH 7.6, 0.25 M sucrose, 0.25 mM EDTA at 37°C.

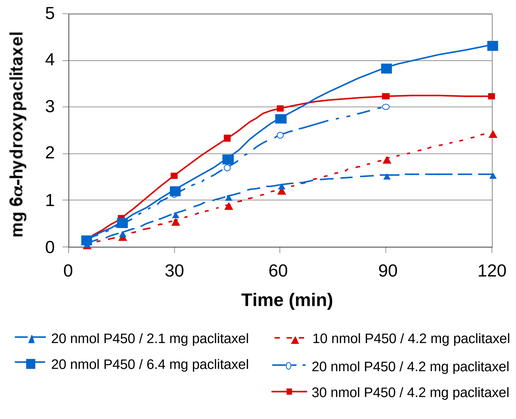
Fig. 3 Overlaid Chromatograms from 5 min and 180 min samples from incubation of CYP2C9 membranes and diclofenac



CYP2C8 / Paclitaxel:

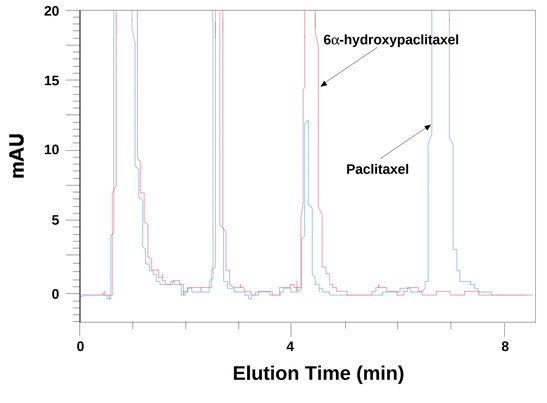
Incubation of paclitaxel with osmotically shocked *E. coli* co-expressing CYP2C8 and NADPH P450 reductase resulted in the near complete conversion of the paclitaxel to its metabolite 6α-hydroxypaclitaxel (Fig. 4). As with CYP2C9 and diclofenac, no detectable additional metabolites were formed during the incubation (Fig. 5). Lower substrate concentrations were used for paclitaxel (maximum 37.5 µM or 34 µg/ml which is 7.5 x Km for the enzyme) because we had previously found evidence of substrate inhibition occurring above 50 µM paclitaxel.

Fig. 4 6α-hydroxypaclitaxel production with *E. coli* containing CYP2C8



Paclitaxel was incubated in 200 ml total volume in 500 ml conical flasks with osmotically shocked *E. coli* expressing CYP2C9 in 50 mM tris-acetate pH 7.6, 0.25 M sucrose, 0.25 mM EDTA at 37°C.

Fig. 5 Overlaid Chromatograms from 5 min and 120 min samples from incubation of *E. coli* containing CYP2C8 and paclitaxel



Conclusion

Here we have shown that recombinant cytochrome P450s can, in principle, be used to manufacture drug metabolites efficiently. The system is easily scalable and the yields are potentially high (in 200 ml the yield was nearly 22 mg 4'-hydroxydiclofenac with 86% of the parent compound converted). When using whole *E. coli* as the source of recombinant enzyme, one of the main costs associated with the production of drug metabolites using recombinant P450s, the provision of NADPH, is removed with little or no reduction in the final yield. We are currently investigating the use of other families of cytochrome P450 (CYP3A and CYP2D) in making metabolites.