

# RAPID IDENTIFICATION AND PRODUCTION OF METABOLITES USING STABILIZED DRIED POWDER (SDP) OF HUMAN CYTOCHROME P450S (hCYP) 2D6, 3A4, AND 2C9 ENGINEERED IN YEAST

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## INTRODUCTION

The larger vision is to metabolically engineer a microbe to “mimic” drug metabolism of human liver. Also, the engineered microbe must be scalable to obtain kilograms of cells within 3-5 days. This will enable biocatalytic prep-scale synthesis of drug metabolites. This innovation is “game-changing” in terms of (i) large scale preparation of drug metabolites for MIST studies and use as standards (ii) drug-drug interaction analysis and (iii) easy identification of human metabolites of drugs for registration. The innovation also has the potential to reduce animal usage due to availability of large amounts of ‘microbial human liver’, to mimic human drug metabolism.

Drug metabolism is a critical aspect of the long and expensive process of drug discovery to market. Generally called ADME (drug absorption, distribution, metabolism, and excretion) and DMPK (drug metabolism and pharmacokinetics), they are key to marketing drugs. Every drug in development needs to be fully characterized with respect to all the metabolites produced in humans. This requires prep-scale synthesis of metabolites, which is usually done by classical synthesis or using animal/human microsomes. This technology is old, cumbersome, difficult, low yielding and time consuming due to the complexity of the metabolite-structure. The biocatalytic tools available to generate mg amount of metabolites are extremely expensive. The present innovation will change this game and make available large amounts of “microbial human liver” for prep-scale synthesis of specific metabolites. The present innovation will also reduce the time and cost of metabolite identification and preparation. This will help pharmaceutical companies make critical decision in terms of identifying the winners in the drug pipeline.

In our present innovation, we have engineered wild type hCYPs and grown in fermenter in several kilograms scale and made SDP mimicking “human liver” reactions.

## ABSTRACT

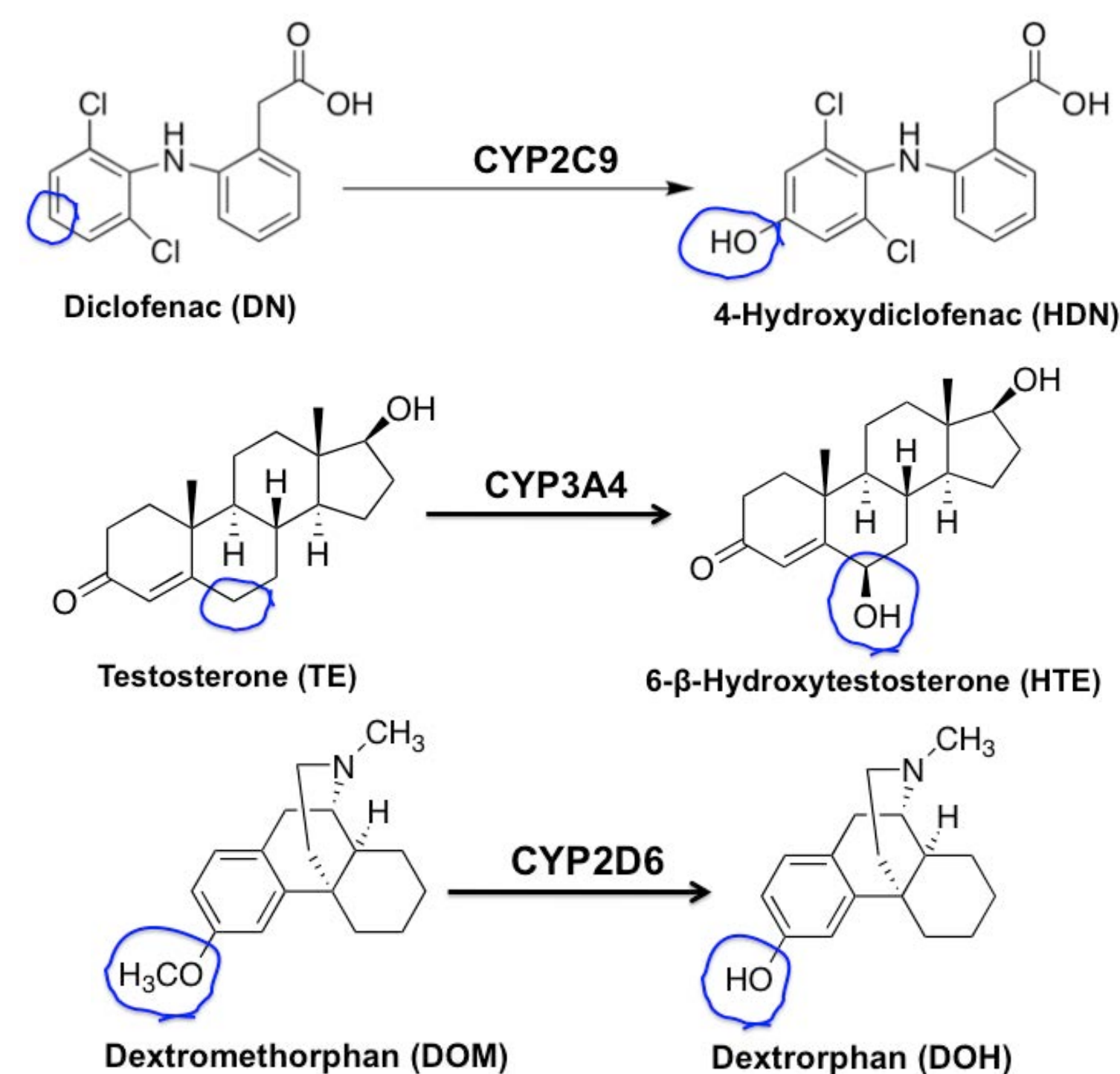
Our larger vision is to metabolically engineer a microbe to “mimic” drug metabolism of human liver. Also, the engineered microbes must be scalable to obtain tens of kilograms of cells within 2-4 days via high cell density fermentation ( $OD_{600}$  around 500). This will simplify and enable biocatalytic prep-scale synthesis of drug metabolites. This innovation is “game-changing” in terms of (i) large scale preparation of drug metabolites for Metabolite in Safety Test (MIST) studies and use as reference standards (ii) drug-drug interaction analysis, and (iii) easy identification of human metabolites of drugs for registration. The innovation also significantly reduces usage of animal-derived materials. Every drug in development needs to be fully characterized with respect to all the metabolites produced in humans. Any metabolite >10% of the drug, must be subject to MIST. This requires prep-scale synthesis of metabolites, which is usually done by classical synthesis or using animal/human microsomes. This technology is old, cumbersome, difficult, low yielding and time consuming due to the complexity of the metabolite-structure. The biocatalytic tools available to generate mg amount of metabolites are extremely expensive. The present innovation changes this game due to availability of large amounts of “microbial human liver” for prep-scale synthesis of specific metabolites. The process is extremely simple; add SDP to drug-candidate of interest. Incubate for 1 to 4 h and analyze metabolites. There is no need for cumbersome microsomal preparation and addition of external NADPH. The present innovation has significantly reduced the hassle, time, and cost of the metabolite identification and preparation. This will help pharmaceutical companies make critical decision in terms of rapid identification of the winners in the drug pipeline.

We have completed the engineering of CYP2D6, CYP3A4, CYP2C9, & CYP2C19 in yeast in our first phase of experiments and made SDP of these CYPs successfully. These SDP preparations mimic “human liver” reactions of corresponding CYPs based on recommended standard substrates for each CYPs. Here, we describe (i) hCYP2C9-SDP catalyzed conversion of diclofenac (DN) to 4-hydroxydiclofenac in microtiter plate, and in 400 mL scale to generate 53.6 mg of HDN from 59.2 mg of DN in only 1.5 hours, and (ii) hCYP3A4-SDP catalyzed conversion of testosterone (TE) to 6 $\beta$ -Hydroxytestosterone (HTE) in microtiter plate and in 200 mL scale. Two cycles of reaction produced 7.01 mg of HTE from 28.8 mg of TE in 3.0 hours. We are currently scaling up other hCYP-SDPs to demonstrate facile synthesis of metabolites in large scale.

## MATERIALS AND METHODS

**Reaction Conditions in Microtiter Plate for Metabolite Identifications:** 250-1000  $\mu$ M Substrate, 20 mg CYP-SDP, 50 mM  $KP_i$  buffer pH 7.5, 30°C, 600 rpm in microtiter plate shaker, Reaction volume 200  $\mu$ L, Reaction time 2 to 4 h

**Large Scale (200 mL) Reaction Conditions for Product Isolations:** 250-500  $\mu$ M Substrate, 20 g washed CYP-SDP, 50 mM  $KP_i$  buffer pH 7.5, final concentration of NaG6P 12 mM, 30°C, 225 rpm in rotary orbital shaker, Reaction volume 200 mL, Reaction time 2 to 4 h



## RESULTS

We have completed the engineering of CYP2D6, CYP3A4, CYP2C9, & CYP2C19 in yeast in our first phase of experiments and made SDP of these CYPs successfully. These SDP preparations mimic “human liver” reactions of corresponding CYPs based on FDA recommended standard substrates for each CYPs. hCYP-SDP catalyzed reactions were carried out in microtiter plate for rapid metabolite identifications. Every reactions were scaled up in several hundreds of milliliter scale to demonstrate the product formation in milligram scale. hCYP2C9-SDP catalyzed conversion of diclofenac (DN) to 4-hydroxydiclofenac in 24-well microtiter plate, produced 398  $\mu$ M of 4-hydroxydiclofenac in 2 hour of reactions. For product isolation, reaction at 400 mL scale was performed and 53.6 mg of HDN from 59.2 mg of DN was generated in only 1.5 hours.

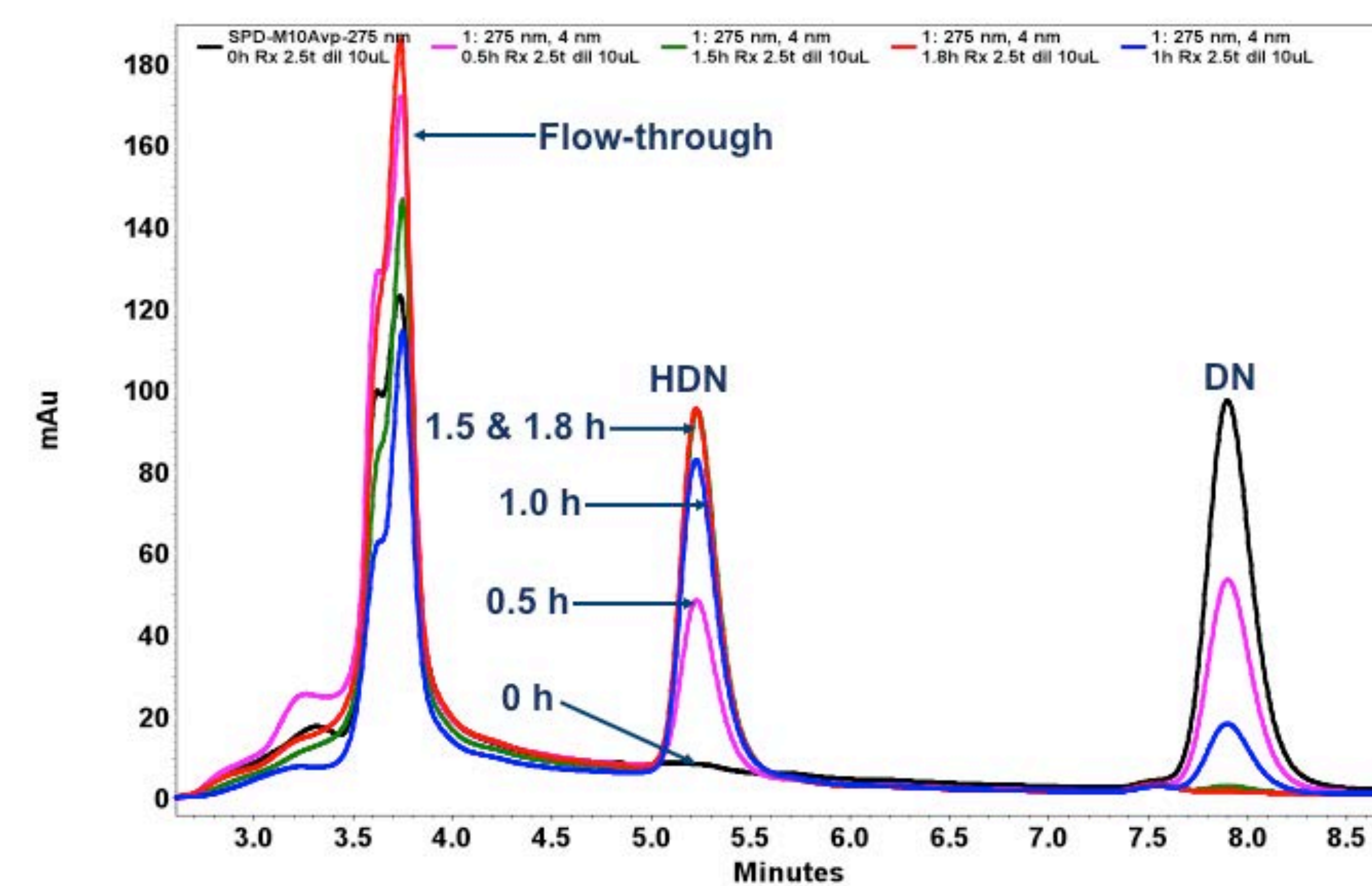
hCYP3A4-SDP catalyzed conversion of testosterone (TE) produced 104  $\mu$ M of 6 $\beta$ -Hydroxytestosterone (HTE) in microtiter plate in 3 h of reactions. For product isolation, this reaction was scaled up in 200 mL. Two cycles of reaction produced 7.01 mg of HTE from 14.4 mg of TE in 3.0 hours.

hCYP2D6-SDP catalyzed conversion of Dextromethorphan (DOM) produced 217  $\mu$ M of Dextrorphan (DOH) in microtiter plate in 4 h of reactions. For the isolation of product DOH, this reaction was scaled up in 200 mL. Two cycles of reaction produced 7.4 mg of DOH from 27.1 mg of DOM in 4.0 hours.

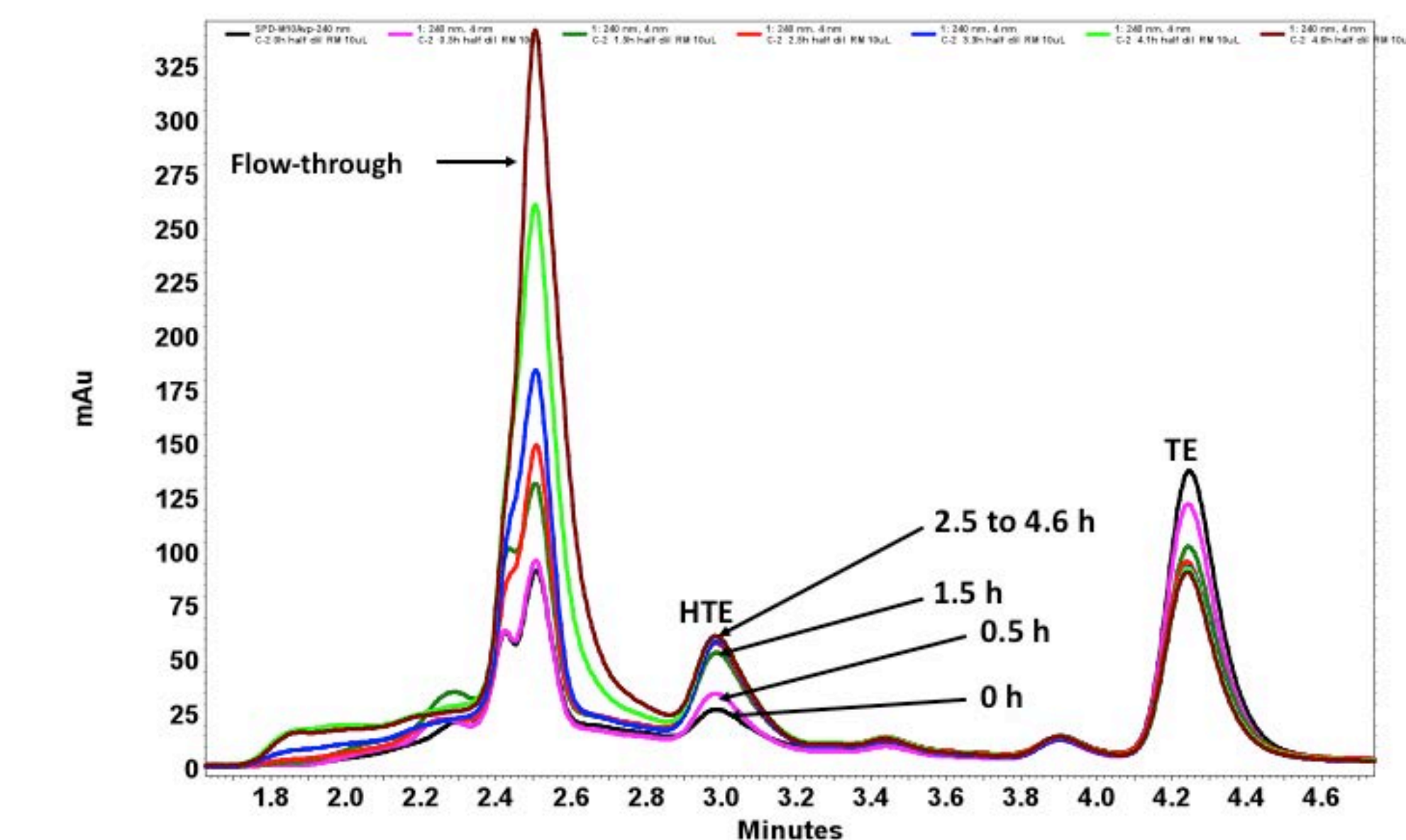
## RB-CYP-SDP CATALYZED CONVERSION AT 24-WELL MICRO-PLATE

No.	CYP	Substrate (Concentration)	Hour of Reaction	Product (Concentration)
1	2C9	Diclofenac (500 $\mu$ M)	2.0	4-hydroxydiclofenac (398 $\mu$ M)
2	3A4	Testosterone (500 $\mu$ M)	3.0	6 $\beta$ -Hydroxytestosterone (104 $\mu$ M)
3	2D6	Dextromethorphan (1000 $\mu$ M)	4.0	Dextrorphan (217 $\mu$ M)

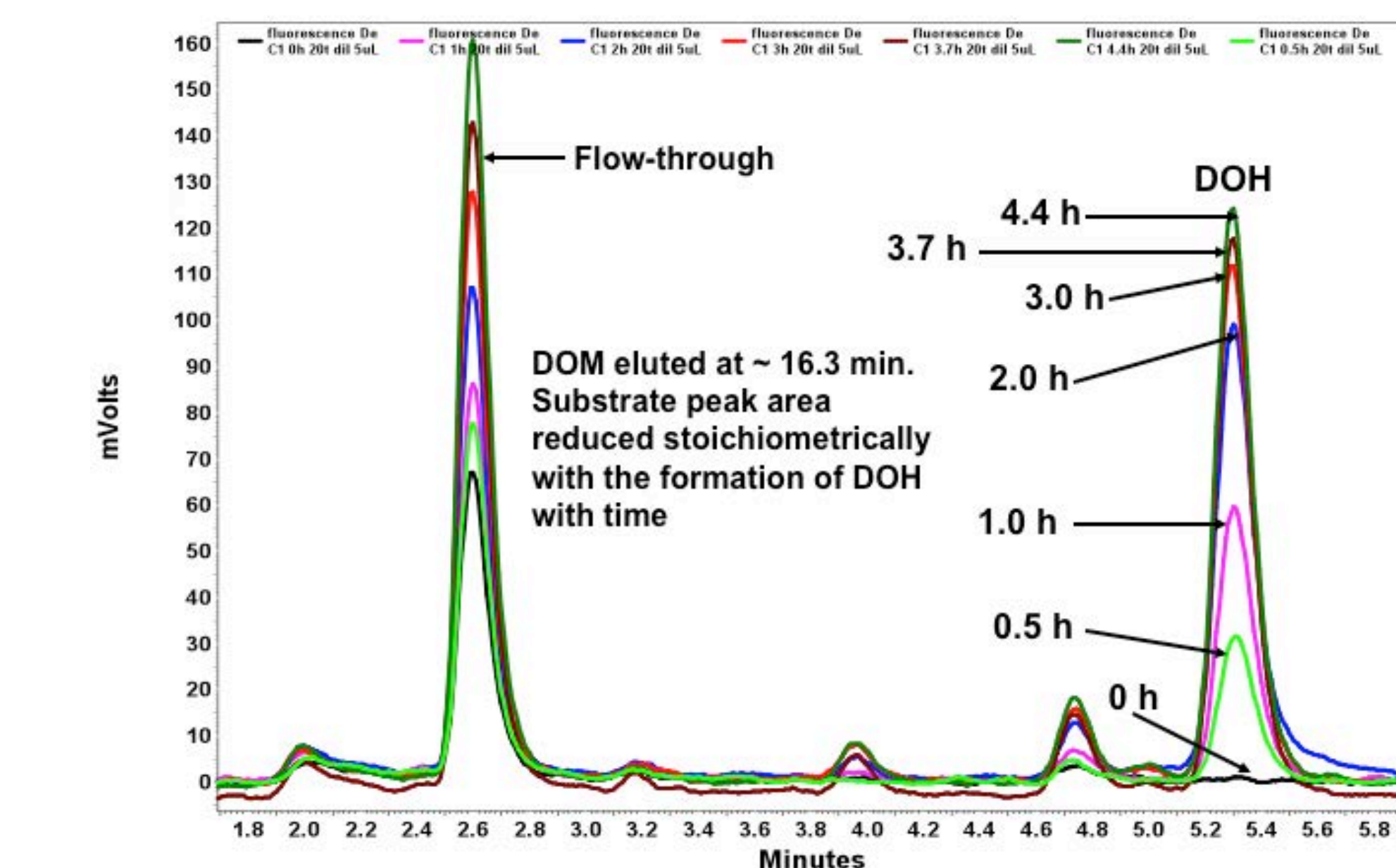
## HPLC PROFILE OF RB-CYP2C9-SDP CATALYZED CONVERSION OF DN TO 4'-HDN AT 400 ML SCALE



## HPLC PROFILE OF CYCLE-1 RB-CYP3A4-SDP CATALYZED CONVERSION OF TE TO 6-HTE AT 200 ML SCALE



## HPLC PROFILE OF CYCLE-1 RB-CYP2D6-SDP CATALYZED CONVERSION OF DOM TO DOH AT 200 ML SCALE



## CONCLUSIONS

✓ Stabilized dry powder containing hCYP is a remarkable substitute of human liver; reaction with SDP is very simple, easy, and inexpensive. Just mixing of drug & SDP in buffer

✓ Metabolite production using SDP is faster and economical over liver microsome or other cloned enzyme

✓ SDP can also be used for rapid metabolite identification in 24-well micro-titer plate

✓ SDP can be prepared in several tens of Kg scale very easily within 3 days which is absolutely impossible for human liver microsomes

✓ SDP can also be used for multiple cycles for production of metabolites for further cost-effective process

✓ No NADPH is required for SDP reactions

## ACKNOWLEDGEMENTS

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