Characterisation of the Induction of Cytochrome p450 Enzymes in Primary Cultures of Human Hepatocytes

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Abstract: The induction of drug metabolising enzymes e.g. cytochrome P450s (CYPs), *in vivo*, by drugs, can attenuate the desired clinical effect or can lead to potential pharmacological effects and/or toxicity. The ability to screen for these undesirable characteristics at an early stage of drug development *ex vivo* is clearly an advantage economically and ethically. Validated methods for the determination of the enzyme activities of a range of CYPs have been established at Covance. This work aims to develop a real time quantitative polymerase chain reaction (RT-QPCR) assay to monitor the induction of individual CYPs in human hepatocytes samples by quantitatively measuring mRNA compared to protein. We have designed Taqman primers and probes for human CYP3A4, CYP2B6 and CYP1A2 and the RT-QPCR method has been developed and validated. Using the validated methods described, the relationship between mRNA expression and enzyme activity will be determined in fresh human hepatocytes.

Key words: Human hepatocytes; Cytochrome P450; Induction; Quantification; Real-time RT-PCR; TaqMan

1. INTRODUCTION

Drug interactions are a major concern both for clinicians and the pharmaceutical industry. A regulatory requirement during the pharmaceutical development process is the *ex vivo* evaluation of the potential of a substance to induce drug metabolising enzymes. *In vivo*

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induction of enzymes which metabolise compounds to inactive products can attenuate the desired clinical effect, whereas increased production of active products or intermediates can lead to potential pharmacological effects and/or toxicity. The ability to screen for undesirable characteristics at an early stage of drug development is clearly an advantage economically and ethically (Bowen *et al.* 2000).

Cytochromes are involved metabolism of various foreign compounds including drugs. It is important to ascertain whether these enzymes are induced or inhibited by a new, potentially therapeutic compound. Various methods have been used in order to measure the expression of cytochromes in biological samples (Rodriguez-Antona *et al.* 1999). However, the use of human hepatocytes has come to be regarded as the 'gold standard' for the *in vitro* quantification of cytochrome P450 (CYP) induction (Roymans *et al.* 2004). Most drugs induce CYP isoforms by upregulating gene transcription, causing increased synthesis of mRNA and enzyme. At present, established enzymatic assays are used at Covance to detect CYP induction. The objective of this study was to develop a real time reverse transcriptase quantitative polymerase chain reaction (RT-QPCR) assay to monitor the induction of individual CYPs in human hepatocyte samples by quantitatively measuring mRNA and comparing this to enzyme activity.

RT-QPCRs to detect the expression of CYP3A4, CYP2B6 and CYP1A2 mRNAs were chosen to be developed initially due to the significance of these CYPs (Plant *et al.* 2003, Faucette *et al.* 2000).

2. MATERIALS AND METHOD

TaqMan primers and probes for human CYP3A4, CYP2B6, CYP1A2 and GAPDH mRNA were designed using the Primer Express software version 1.1 (Applied Biosystems). The primers and probes were designed respectively from the NCBI database sequences NM_017460, NM_000767, NM_000761 and NM_002046.

Assays were performed using the Applied Biosystems ABI Prism® 7700 sequence detection system. Using human liver Total RNA (Ambion®) to generate a standard curve, RT-QPCRs for CYP3A4, CYP2B6 and CYP1A2 mRNA relatively efficient to the RT-QPCR for the endogenous control, GAPDH mRNA, were developed following the principles detailed in the ABI Prism® 7700 Sequence Detection System User Bulletin #2. Following this, the RT-QPCR methods for GAPDH, CYP3A4, CYP2B6 and CYP1A2 were then developed and validated in the spirit of the ICH guideline on the validation of analytical procedures, QB2.

3. RESULTS

Table 1 summarises the results obtained from the development and validation.

Table 1. Results Summary. The results were considered acceptable if less than 6%.

	GAPDH	CYP3A4	CYP2B6	CYP1A2
Relative Efficiency	N/A	10 – 1x10^6 pg	1 – 1x10^6 pg	10 – 1x10^5 pg
Specificity	Human Mouse	Human	Human Mouse	Human
Linearity and Range	1 – 1x10^4 pg	1 – 1x10^5 pg	10 – 1x10^5 pg	100 – 1x10^5 pg
Accuracy	N/A	N/A	N/A	N/A
Precision	<2.24%	<3.44%	<5.30%	<2.15%
Quantification Limit	1pg	1pg	10pg	10pg
Operator Robustness	<1.82%	<3.61%	<3.44%	<2.28%
Reagent Robustness	<1.59%	<3.00%	<2.27%	<0.88%

4. DISCUSSION

The methodology used for the amplification and detection of GAPDH, CYP3A4, CYP2B6 and CYP1A2 mRNAs was deemed to be validated.

For measuring cytochrome P450 induction, the range of hepatocyte total RNA to be used will be 10-1x10^4, 10-1x10^4 and 100-1x10^4 pg for the detection of CYP3A4, CYP2B6 and CYP1A2 induction respectively.

Work has begun on in vitro cytochrome P450 induction in human hepatocytes using the inducers rifampicin (CYP3A4), omeprazole (CYP2B6) and phenobarbital (CYP1A2) with enzyme activity quantified using the selective substrates testosterone (CYP3A4), bupropion (CYP2B6) and ethoxyresorufin (CYP1A2). mRNA analysis using the RT-QPCRs described here will be carried out in parallel and the results generated compared with the enzyme activity data.

Total RNA extraction methods have been evaluated.

Preliminary work has shown that the RT-QPCRs have been successful in detecting the cytochrome P450 mRNA extracted from human hepatocytes cultured in our laboratories (results not shown). In addition to detecting mRNA, the RT-QPCRs for GAPDH and CYP2B6 also appeared to detect DNA. This is believed to be due to inadequate DNA removal during the RNA extraction. In these assays, detection of DNA is possible due to the amplicon size, whereas the assays for CYP3A4 and CYP1A2 have larger amplicons and therefore are not detected using the current thermal cycler conditions.

REFERENCES

Bowen WP, Carey JE, Miah A, McMurray HF, Munday PW, James RS, Coleman RA and Brown AM (2000) Measurement of Cytochrome P450 Gene Induction in Human Hepatocytes using Quantitative Real-time Reverse Transcriptase-Polymerase Chain Reaction. Drug Metab and Dispos **28:**781-788

Rodriguez-Antona C, Jover R, Gomez-Lechon M and Castell JV (1999) Quantitative RT-PCR Measurement of Human Cytochrome P-450s: Application to Drug Induction Studies. Arch Biochem Biophys **376:**109-116

Roymans D, Van Looveren C, Leone A, Brandon Parker J, McMillian M, Johnson MD, Koganti A, Gilissen R, Silber P, Mannens G and Meuldermans W (2004) Determination of Cytochrome P450 1A2 and Cytochrome P450 3A4 Induction in Cryopreserved Human Hepatocytes. Biochem Pharmacol **67:**427-437

Plant NJ and Gibson GG (2003) Evaluation of the Toxicological Relevance of CYP3A4 Induction. Curr Opin Drug Discov Devel **6:**50-56

Faucette SR, Hawke RL, Lecluyse EL, Shord SS, Yan B, Laetham RM and Lindley CM (2000) Validation of Bupropion Hydroxylation as a Selective Marker of Human Cytochrome P450 2B6 Catalytic Activity. Drug Metab Dispos **28:**1222-1230

ABI Prism® 7700 Sequence Detection System User Bulletin #2 on Relative Quantification of Gene Expression

International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) (1996) Guidance for Industry. QB2 Validation of Analytical Procedures: Methodology. Food and Drug Administration Federal Register: May 19, 1997 (62 FR 27464)