Screening for Drug-Drug Interactions Using a Targeted Proteomics Strategy

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Overview

- Drug-drug interactions can often be measured by CYP induction and inhibition studies.
- This poster describes the use of a proteomics method to measure the extent of CYP induction.
- The levels of CYP proteins from induction studies were determined based on the levels of their surrogate tryptic peptides.
- Quantitation of peptides was accomplished using scheduled MRM including the corresponding heavy peptide analogs as internal standards.
- Induction results obtained this way were compared to the more traditional CYP activity assay utilizing probe substrates.

Introduction

- Cytochrome P450 proteins (CYPs) are major drug metabolizing enzymes responsible for phase I biotransformations. For pharmaceutical companies, measurement of CYP induction in response to a drug candidate is a fundamental aspect of assessing drug-drug interactions, and evaluating drug safety and efficacy.
- There are currently three main methodologies for assessing CYP450 induction: mRNA measurements, enzymatic activity measurements, and Western blot analysis.
- Although all three are widely used throughout the pharmaceutical industry, there are limitations to each. mRNA data, for example, may be influenced by non-transcriptional mechanisms; enzymatic activity measurements may be affected by non-linearity of the assay and availability of probe substrates; and the drawback of Western blot analysis is usually caused by cross-reactivity of isoform-specific antibodies.
- To overcome these limitations and improve the understanding of CYP induction from drug candidates, it is advantageous to directly measure protein expression changes of individual CYP450 isoforms with high specificity, sensitivity, and accuracy.
- Recently, a robust LC/MS method to quantify the protein levels of four key CYP450 protein isoforms was established for induction studies: 1A2, 2B6, 3A4, and 3A5.
- Highly selective and sensitive tryptic peptides were selected for each of the CYP isoforms, enabling the targeted proteomic analysis. A set of isotopically enriched peptides are used for performing relative quantitation experiments on four CYP isoforms.
- Correlation between enzymatic activity, mRNA expression and protein expression measured by LC/MS, have been assessed previously for a small set of known inducers (e.g., rifampin, 3-Methylcholanthrene and Phenobarbital).
- The observed changes agreed well with results for incubation of hepatocytes with these known inducers. Overall, good correlation between total protein increase, enzyme activity and mRNA levels was observed (**Figure 1**).
- We have recently made improvements to the technology that enhance both sensitivity and selectivity of detection as well as expand the number of CYP enzymes capable of being evaluated simultaneously to include CYP2C19, CYP2E1 and CYP2C9.
- We have also expanded on this validation set to include a wider range of known, published inducers.

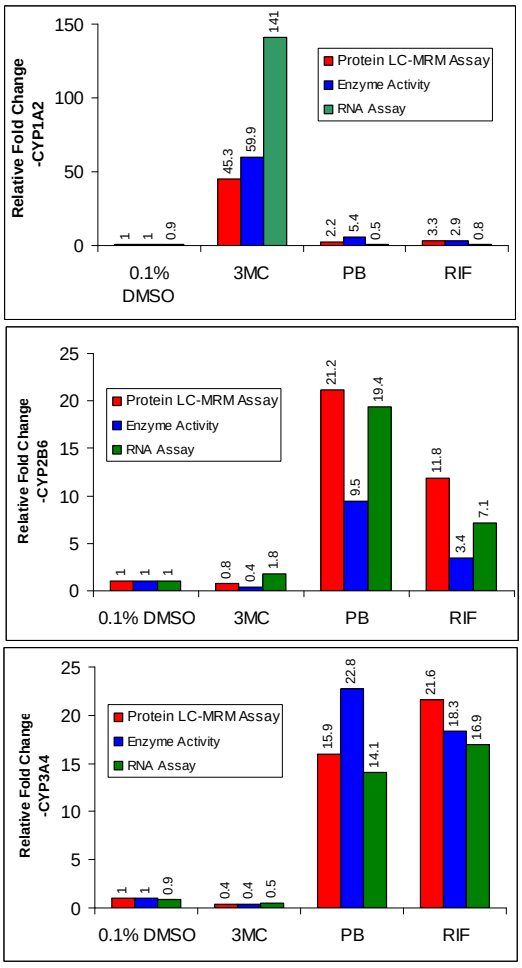


Figure 1. Comparing Protein, mRNA and Enzyme Activity in the Induction Controls. Protein expression changes measured by LC-MRM analysis agree the changes observed in mRNA assays and enzyme activity. (Top) CYP 1A2 showed induction by 3-methylcholanthrene (3MC) as expected. (Middle) CYP 2B6 was induced by both phenobarbital (PB) and rifampin (RIF). (Bottom) CYP 3A4 was also induced by both PB and RIF.

Method and Instrumentation

- S9 or microsomal subcellular fractions, prepared by lysing the cryopreserved hepatocytes and performing one or more steps of centrifugation, were used for these studies.
- To facilitate analysis a minimum of 2 x 10^6 cells per analysis was used (250 ug total protein).
- In addition to Rifampin, Phenobarbital, Omeprazol and Isoniazid were also used as inducers. Activity assays were conducted in the presence of phenacetin (1A2), Diclofenac (2C9), Mephenytoin (2C19), Chlorzoxazone (2E1), Midazolam and Testosterone (3A4) as probe substrates.
- Improvements in sample processing (as shown in **Figure 2A)** included: 1. Sample cleanup using sandwiched 100K and 10K membrane filters; 2. Enhancement in sample digestion using a Barocycler® NEP2320.
- Each sample was reduced, alkylated, and digested with trypsin (**Figure 2B**). The CYP450 Human Induction Kit (AB Sciex) was used for sample preparation. Time of sample preparation was about 4 hours including tryptic digestion. Samples were analyzed using an API4000 QTrap.
- The scheduled MRM (sMRM) methods were initially developed by Midas workflow and later adapted and optimized for UHPLC/MS system. One hundred and fifty eight transitions were monitored within a 28min UHPLC run. Both the light peptides and heavy internal standards were monitored (**Figure 3**).
- Data was processed with MultiQuant™ Software and Microsoft Excel. Protein expression changes were automatically computed and graphically displayed.

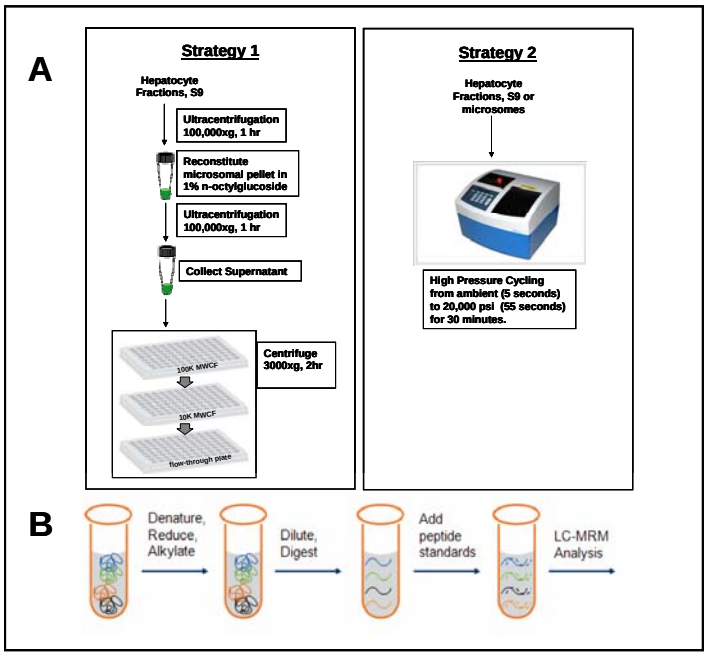


Figure 2. (A) Sample cleanup. Strategy 1 employed MW cut off membrane filters to isolate the CYP enzymes, and remove the unwanted proteins to reduce sample complexity. Strategy 2 employed high pressure cycling for more efficient protein digestion. **(B) Sample Preparation Protocol.** Protein fraction was denatured, then disulfide bridges were reduced and alkylated. Solution was diluted to reduce the denaturant concentration before protein digestion. Trypsin was added and the proteins were digested. The stable isotope labeled peptide internal standards (heavy peptide solution) were added and sample was submitted for LC-MS analysis.

Q1	Q3	DP	CE	CXP	Q1	Q3	DP	CE	CXP	Q1	Q3	DP	CE	CXP	Q1	Q3	DP	CE	CXP	Q1	Q3	DP	CE	CXP
569.4	566.6	50	22	15	419.2	603.5	50	20	15	553.5	576.4	60	35	15	469	742.7	60	24	15	472.3	589.3	55	28	15
569.4	720.4	50	31	15	419.2	690.6	50	23	15	553.5	691.3	60	23	15	464	342.3	60	24	12	472.3	686.4	55	22	15
565.5	561.6	50	22	15	594	678.4	70	32	15	553.5	921.4	60	23	15	464	585.5	60	24	15	472.3	743.5	55	20	15
565.5	710.4	50	31	15	594	778.7	70	24	15	548.3	566.3	60	35	15	464	732.7	60	24	15	468.3	735.5	55	20	15
853.2	1131.9	90	44	15	594	1164.8	70	34	15	548.3	681.3	60	23	15	709.7	972.8	65	28	15	468.3	678.5	55	22	15
853.2	1230.8	90	43	15	590.5	668.4	70	32	15	548.3	911.4	60	23	15	709.7	1120.7	65	31	15	468.3	581.4	55	28	15
848.1	1121.9	90	44	15	590.5	768.7	70	24	15	426.2	518.4	70	23	15	709.7	1191.9	65	31	15	592	650.5	60	20	15
848.1	1220.8	90	43	15	590.5	1154.8	70	34	15	426.2	617.3	70	22	15	705.7	964.8	65	28	15	592	700	60	21	15
605.1	611.5	80	34	15	890	225.4	100	55	10	426.2	704.4	70	24	15	705.7	1112.7	65	31	15	592	749.5	60	19	15
600.1	601.5	80	34	15	885	225.4	100	55	10	421.2	508.4	70	23	15	705.7	1183.9	65	31	15	589.1	646.5	60	20	15
403.9	369.3	50	19	10	333.9	252.3	40	17	10	421.2	607.3	70	22	15	854.8	1121.6	95	43	15	589.1	696	60	21	15
403.9	498.3	50	18	15	333.9	415.3	40	14	15	421.2	694.4	70	24	15	854.8	1223	95	41	15	589.1	745.5	60	19	15
403.9	598.4	50	17	15	333.9	466.4	40	14	15	444.9	542.3	50	21	15	854.8	1335.6	95	43	15	436.7	644.4	55	20	20
403.9	580.5	50	18	15	333.9	579.4	40	16	15	444.9	559.3	50	21	15	850.8	1113.6	95	43	15	436.7	543.3	55	20	20
400.5	359.3	50	19	10	329.9	244.3	40	17	10	444.9	660.4	50	21	15	850.8	1215	95	41	15	436.7	302.2	55	30	19
400.5	488.3	50	18	15	329.9	415.3	40	14	15	439.7	532.3	50	21	15	850.8	1327.6	95	43	15	432.7	636.4	55	20	20
400.5	598.4	50	17	15	329.9	458.4	40	14	15	439.7	549.3	50	21	15	570.3	564.2	50	14	15	432.7	535.3	55	20	20
400.5	580.5	50	18	15	329.9	571.4	40	16	15	439.7	650.4	50	21	15	570.3	558.3	50	16	15	432.7	294.2	55	30	19
627.7	542.7	55	24	15	456.9	372	45	17	10	567.3	693.4	60	23	15	570.3	612	50	20	15	532.7	622.4	70	26	15
627.7	710.5	55	28	15	456.9	498.4	45	30	15	567.3	750	60	23	15	570.3	668.7	50	20	15	532.7	509.2	70	27	15
627.7	824.6	55	28	15	456.9	595.5	45	18	15	567.3	793.5	60	23	15	570.3	775.8	50	18	15	532.7	735.4	70	25	15
627.7	971.7	55	25	15	456.9	742.6	45	17	15	564.3	689.4	60	23	15	567.6	564.2	50	14	15	528.7	614.4	70	26	15
627.7	1084.7	55	28	15	451.9	367	45	17	10	564.3	745.9	60	23	15	567.6	558.3	50	16	15	528.7	501.2	70	27	15
623.7	538.7	55	24	15	451.9	488.4	45	30	15	564.3	789.5	60	23	15	567.6	608	50	20	15	528.7	727.4	70	25	15
623.7	702.5	55	28	15	451.9	585.5	45	18	15	801	827.4	80	28	15	567.6	664.7	50	20	15	541.3	805.4	70	26	18
623.7	816.6	55	28	15	451.9	732.6	45	17	15	801	940.5	80	29	19	567.6	771.8	50	18	15	541.3	594.3	70	32	18
623.7	963.7	55	25	15	483.5	507.3	55	22	15	801	1011.5	80	29	19	473.5	502.4	55	25	12	541.3	708.4	70	35	18
623.7	1076.7	55	28	15	483.5	622.3	55	22	15	798.4	819.4	80	28	15	473.5	616.4	55	21	15	536.3	795.4	70	26	18
424.2	500.4	50	22	15	483.5	735.4	55	22	15	798.4	932.5	80	29	19	473.5	729.5	55	19	15	536.3	584.3	70	32	18
424.2	613.5	50	20	15	479.2	499.3	55	22	15	798.4	1003.5	80	29	19	469.5	494.3	55	25	12	536.3	698.4	70	35	18
424.2	700.6	50	23	15	479.2	614.3	55	22	15	469	342.3	60	24	12	469.5	608.3	55	21	15					
419.2	490.4	50	22	15	479.2	727.4	55	22	15	469	595.5	60	24	15	469.5	721.4	55	19	15					

Figure 3. MRM transitions for the detection and quantification of heavy and light peptides. In general a minimum of 3 peptides for each CYP isozyme were selected as surrogates, and a minimum 3 MRM transitions were selected from each peptide to be included for the creation of scheduled MRM method. Pink = 2E1, Orange = 2C9, Yellow = 2B6, Green = 3A4, Blue = 2C19, Grey = 3A5, Purple = 1A2

Results

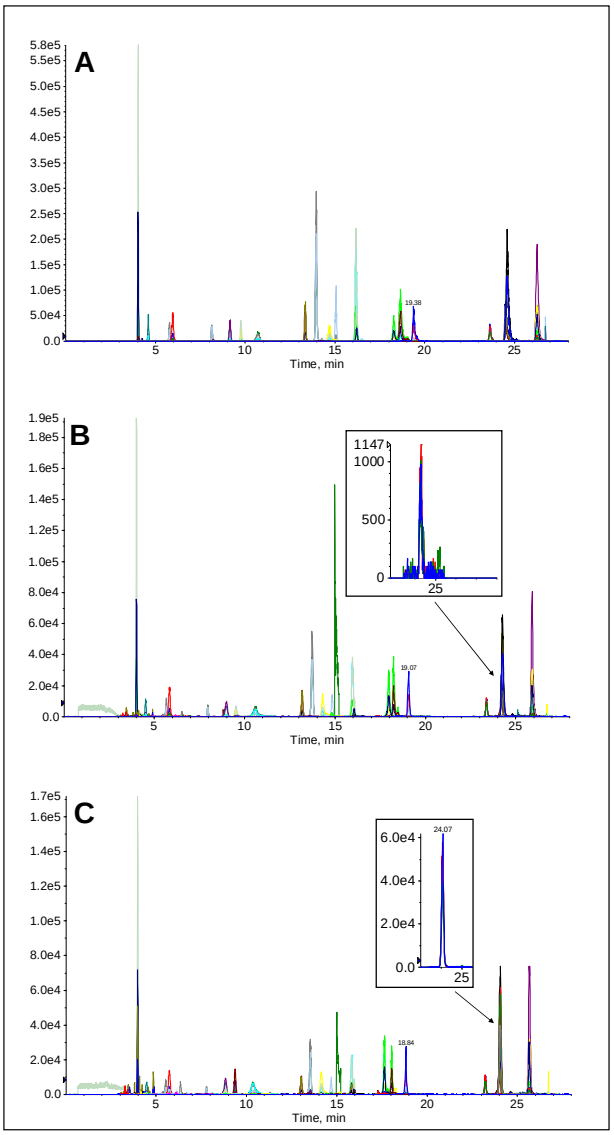


Figure 4. (A) Heavy peptide standards by LC/MS with scheduled MRM detection. Typical results for control and induced hepatocytes samples are shown in **(B)** and **(C)** respectively. The levels of one of the 3A4 peptides were also highlighted in the inserts, showing increasing level in the sample induced with rifampin.

Results (cont.)

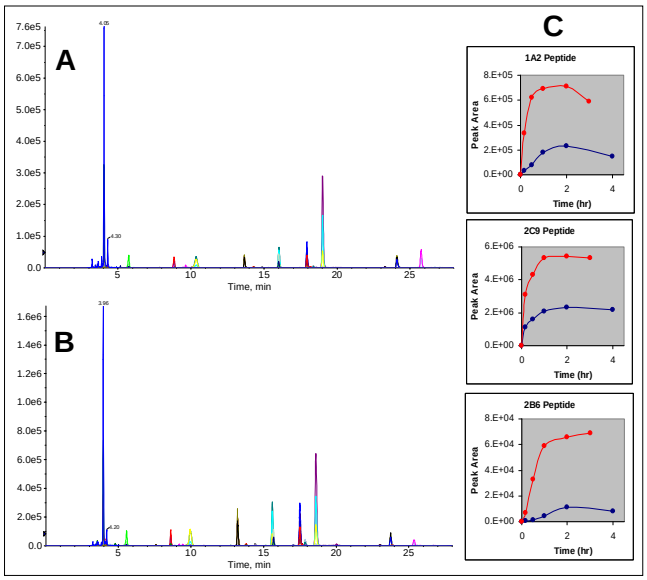


Figure 5. Comparison of detection sensitivity for samples with and without high pressure cycling. **(A)** 1 mg/ml microsomal sample without pressure cycling, digested for 2 hrs. at 37°C **(B)** 1 mg/ml microsomal sample with pressure cycling, digested for 30 min. at 37°C **(C)** Time course tryptic digest with pressure cycling (red) and without pressure cycling (blue) of selective CYP isozymes.

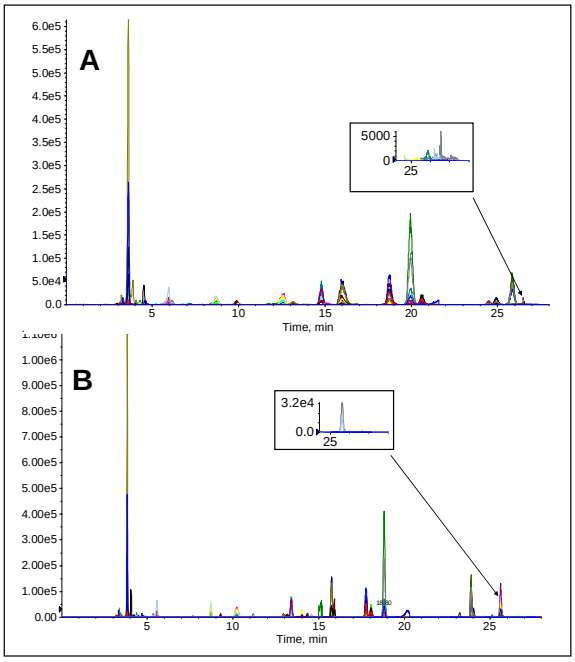
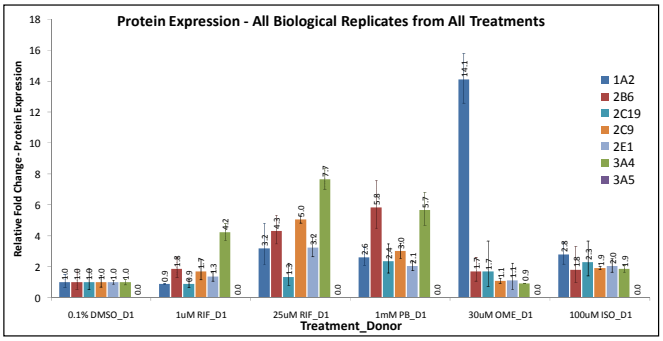


Figure 6. Comparison of detection sensitivity for samples analyzed by HPLC and UHPLC methods. **(A)** HPLC 28 minute separation, AB Sciex Peptide C18 5µm 50x2.1mm, Room Temperature **(B)** UHPLC 28 minute separation, Agilent Eclipse Plus C18 1.8um 100x2.1mm, 50°C. The levels of a less sensitive peptide are highlighted in the inserts.

Conclusions

- The combined used of pressure cycling and UHPLC results in the improvement of the detection sensitivity of the CYP peptides making this protein-LC/MRM detection method as cost effective as the traditional enzyme activity measurements for 7 CYPs.
- Results obtained from proteomics approach were comparable to those obtained by activity assays.
- One unique feature for the proteomics approach is that it allows CYP induction assay to be performed across multiple species when appropriate surrogate peptides that are common to the species of interest are used in MS detection. This will be considered for future works.

Results (cont.)



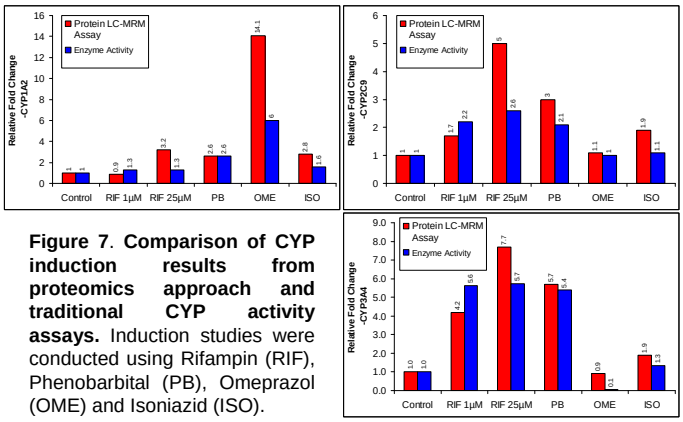


Figure 7. **Comparison of CYP induction results from proteomics approach and traditional CYP activity assays.** Induction studies were conducted using Rifampin (RIF), Phenobarbital (PB), Omeprazol (OME) and Isoniazid (ISO). Protein expression changes measured by LC-MRM analysis and enzyme activity measured using probe substrates, Phenacetin for 1A2, Midazolam for 3A4 and Diclofenac for CYP2C9.

Discussion

- One of the main objectives was to improve the detection sensitivity of the CYP peptides over the existing approach so that we could reduce the number of hepatocyte cells used in the induction assay, which ultimately reduces the cost of the assay.
- The strategies attempted included 1. sample cleanup step utilizing molecular weight cut off membrane; 2. improved sample digestion yield and efficiency utilizing pressure cycling technology; 3. better peptide chromatography utilizing UHPLC technology.
- The molecular weight cut off approach was not effective in improving detection sensitivity; a significant sample loss was observed after membrane filter treatment probably due to the hydrophobic nature of the CYP proteins.
- The use of pressure cycling and UHPLC however have resulted in improvement in peptide detection sensitivity; higher tryptic peptide yield was observed when the digestion was conducted at 20,000psi, and higher peak resolution and sensitivity were observed with UHPLC.
- Activity assay results for CYP3A5 were unavailable due to a lack of an appropriate probe substrate. Basal levels of protein were not detected by the proteomics approach for CYP3A5.
- The proteomics approach allowed detection of CYP2E1 and CYP2C19 induction which was not captured by enzyme activity. Formation of chlorzoxazone-6-hydroxylation is highly sensitive to amounts of solvent used; therefore, affecting enzyme activity detection. CYP2C19 activity was too low to detect the mephenytoin 4-hydroxylation in cultured hepatocytes.

Acknowledgements

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