THE DEVELOPMENT OF AN MRM ASSAY FOR QUANTITATION OF LOW ABUNDANCE CYTOCHROME P450 PROTEINS

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INTRODUCTION

The verification and validation of protein markers identified from the discovery phase of a proteomics experiment typically involves the detection, identification and quantitation of multiple proteotypic peptides. Given that, in any one study, there may be numerous samples containing large numbers of proteins, a holistic approach to identification and subsequent quantification is advantageous.

Cytochromes P450 (CYP450) are a large group of heam containing proteins that reside within the cell's endoplasmic reticulum. CYP450s are extensively studied due to their major role in oxidative metabolism and their potential to be induced or inhibited by a wide range of drugs, foods and disease states. The ability to quantify and monitor levels of specific CYP450 enzymes is a useful therapeutic tool. However, high sequence homology between CYP isoforms can make this a challenging task.

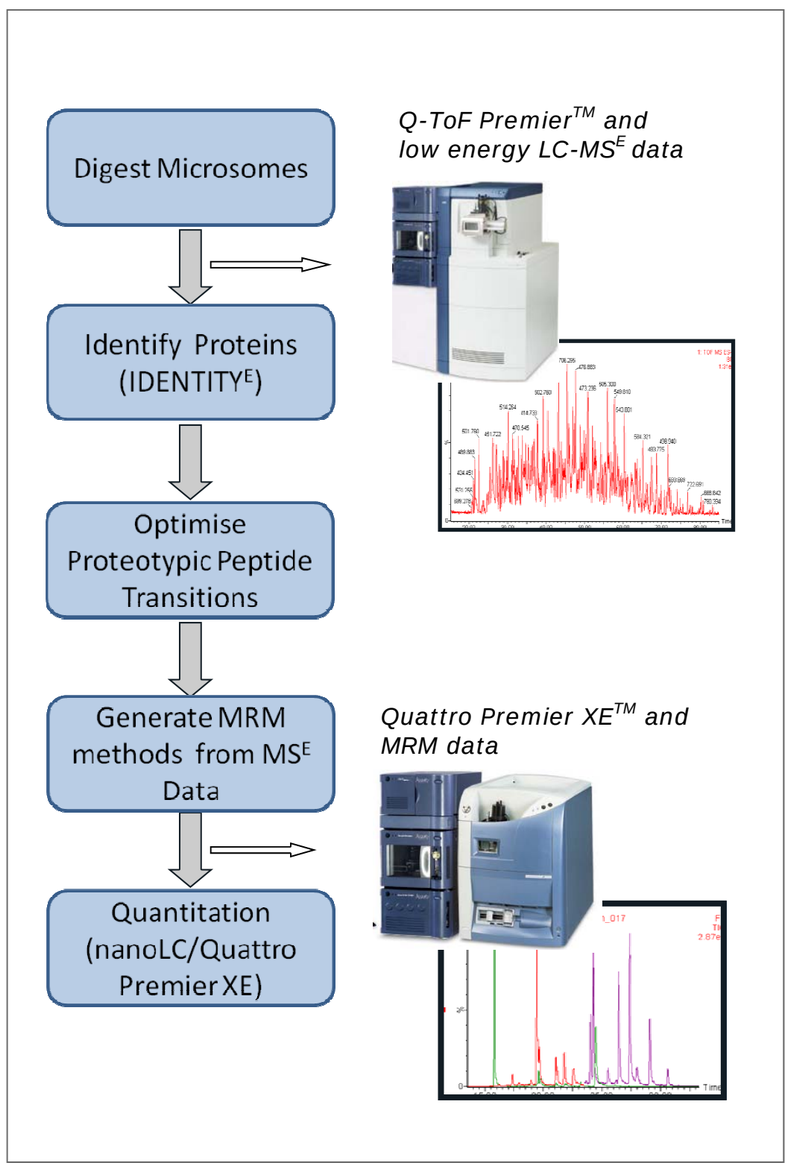
This study describes the complete workflow, from protein identification through to peptide quantification. Here the workflow is applied to the identification and then absolute quantification (against specific stable isotopically labeled peptides) of key CYP450 enzymes in chemically induced rat microsomes (liver cells). The induced microsomes should display up or down regulation of the CYP450 enzymes compared to a control (see figure 1).

Using the IDENTITY^E high definition proteomics system, CYP450 proteins, in a control rat microsomal tryptic digest, were confidently identified from their tryptic peptides. LC-MS^E data from this analysis provided precursor and fragment ion information, including intensity, which was then systematically filtered through a series of criteria to optimise parameters for quantification. Multiple reaction monitoring (MRM) methods were automatically built from the sorted IDENTITY^E data. Finally, the selected MRM methods were implemented on a triple quadrupole mass spectrometer and used for absolute quantification of the peptides using stable isotope dilution.

Chemical Inducer	Main Induced CYP450
CONTROL	
AROCLOR_1254	1A2
NAPTHOFLAVONE	1A2
DEXAMETHAHSONE	3A1
3-METHYLCHLOANTHERE	2A1
PHENOBARBITAL	2B2 and 2C6 and 3A2

Figure 1. The Key CYP450s induced in the Chemically Induced Rat Liver Microsomes used in this study.

PEPTIDE QUANTIFICATION METHOD DEVELOPMENT WORKFLOW



METHODS

Microsomal Protein digest

100µg of control or induced rat liver microsomal protein (Celsis International Plc, Cambridge, UK) was denatured in 0.1% RapiGest™ (Waters,Milford ,MA), before reduction with DTT (100mM) and alkylation with IA (200mM). 1:50 (w/w) of Trypsin (Promega) was added prior to overnight incubation for digestion of the microsomal proteins. Digestion was quenched by the addition of 1µL HCL followed by centrifugation. The final protein concentration was 7.14µg/µl.

Samples

Induced microsomal digest supernatant was diluted 1/50 in 0.1% formic acid containing 5fm/µl of each custom labelled peptide (Sigma-Aldrich , all > 97% purity). Samples were prepared in triplicate to assess reproducibility.

Standards

Labelled peptides were serially diluted and spiked into a 1/50 dilution of control microsomal protein digest of to give a final calibration range of 0.05-50fm/µL. The standard curve was injected in duplicate.

DATA ACQUISITION AND PROCESSING

MASS SPECTROMETRY

Waters® IDENTITY^E system comprising; a Waters Q-Tof Mass Spectrometer, nanoACQUITY and PLGSv2.3, was used for protein identification. LC-MS^E acquisition is an alternating scanning approach that provides exact mass data for each detectable peptide ion in the low energy function and CID fragmentation information in the elevated energy scan, all from a single injection. Peak detection and time alignment of precursor peptides to fragments were performed to assign the deconvoluted mass, retention time, intensity and fragment ion information for each detected species. Database searching was carried out against the Rat Uni-prot database, (www.uniprot.org) using mass tolerances of 10ppm on the precursor and 20ppm on the associated fragments.

MRM transitions were acquired using a Waters Quattro Premier XE triple quadrupole Mass Spectrometer. Specific, optimised MRM transitions for each peptide were obtained from the LC-MS^E data acquired on the Q-ToF instrument. Two MRM transitions were acquired for each endogenous and labelled Cyp450 protein peptide (one peptides per protein) and the MRM channels were divided into three time defined functions to maximise the number of data points acquired across each peak (at least 50).

CHROMATOGRAPHY

1µL of digest was injected onto a 180µmx20mm Symmetry 5µm C_{18} trap column for desalting, followed by separation on a 75µmx250mm BEH130 C_{18} UPLC column or 75µmx100mm column (MRM). Peptide elution was obtained by means of an 0.1% Formic acid/Acetonitrile gradient over 100 minutes for the IDENTITY^E analysis. For the MRM analysis the gradient was shortened to 32 minutes using the above mobile phases.

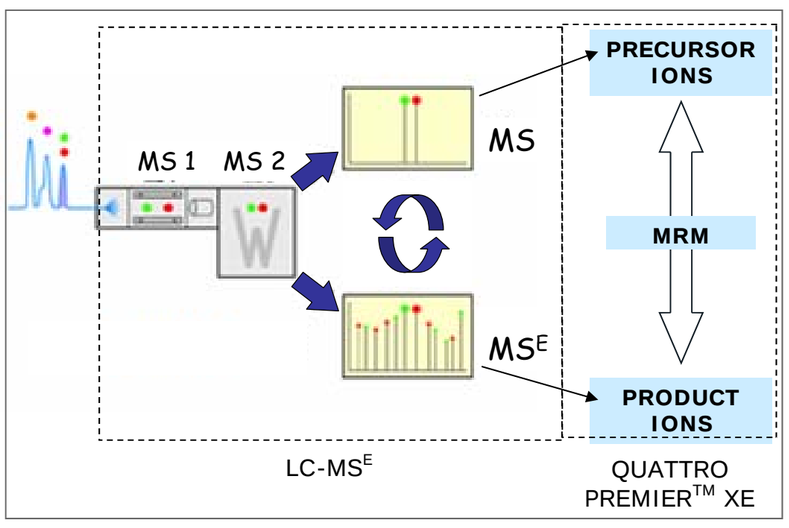


Figure 2. LC-MS^E acquires low energy and elevated energy information in a parallel manner. LC-MS^E acquisition produces an inventory of all detectable precursor and fragment ions through the entire chromatographic run, along with their corresponding intensities . This information is sorted to obtain optimal proteotypic MRM transitions for a tandem quadrupole acquisition.

RESULTS

CYP450	Transition	Sequence	Precursor m/z (z=2)	Product m/z (z=1)	Cone Voltage (V)	Collision Energy (eV)
2A2	Primary	IVVLYGYDAVK	620.4	1027.5	37	25
	Confirmatory	IVVLYGYDAVK	620.4	928.5		
	Primary (Labelled)	IVVLYGYDAV**[K]**	624.6	1035.9		
	Confirmataory (Labelled)	IVVLYGYDAV**[K]**	624.6	936.6		
2A1	Primary	VVVLYGYDAVK	613.3	1027.5	35	20
	Confirmatory	VVVLYGYDAVK	613.3	928.5		
	Primary (Labelled)	VVVLYGYDAV**[K]**	617.4	1035.9		
	Confirmataory (Labelled)	VVVLYGYDAV**[K]**	617.4	936.1		
2C11	Primary	VQEEIER	451.7	675.3	30	18
	Confirmatory	VQEEIER	451.7	786.4		
	Primary (Labelled)	VQEEIE**[R]**	456.9	685.3		
	Confirmataory (Labelled)	VQEEIE**[R]**	456.9	654.5		
3A2	Primary	LQEEIDGALPSK	650.3	1058.5	40	25
	Confirmatory	LQEEIDGALPSK	650.3	800.5		
	Primary (Labelled)	LQEEIDGALPS**[K]**	654.5	1067.8		
	Confirmataory (Labelled)	LQEEIDGALPS**[K]**	654.5	808.4		
2E1	Primary	EAQFLVEELK	603.3	617.4	38	20
	Confirmatory	EAQFLVEELK	603.3	730.4		
	Primary (Labelled)	EAQFLVEEL**[K]**	607.5	625.2		
	Confirmataory (Labelled)	EAQFLVEEL**[K]**	607.5	738.3		
2C7	Primary	GFGIVFSNGNR	584.8	964.5	35	20
	Confirmatory	GFGIVFSNGNR	584.8	907.5		
	Primary (Labelled)	GFGIVFSNGN**[R]**	587.6	970.3		
	Confirmataory (Labelled)	GFGIVFSNGN**[R]**	587.6	913.5		
2C6	Primary	GSFPVAEK	417.7	543.3	25	15
	Confirmatory	GSFPVAEK	417.7	446.3		
	Primary (Labelled)	GSFP**[V]**AEK	421.9	551.3		
	Confirmataory (Labelled)	GSFP**[V]**AEK	421.9	454.3		
1A2	Primary	NSIQDITGALFK	653.9	992.5	40	22
	Confirmatory	NSIQDITGALFK	653.9	864.5		
	Primary (Labelled)	NSIQDITGALF**[K]**	658.2	1000.6		
	Confirmataory (Labelled)	NSIQDITGALF**[K]**	658.2	872.5		

Figure 3. Summary of the ion inventory from the discovery data for the endogenous CYP450 proteins, after filtering using a series of criteria. Final proteotypic peptides (precursor and fragment ions) are displayed with labelled peptide MRM transitions for quantification.

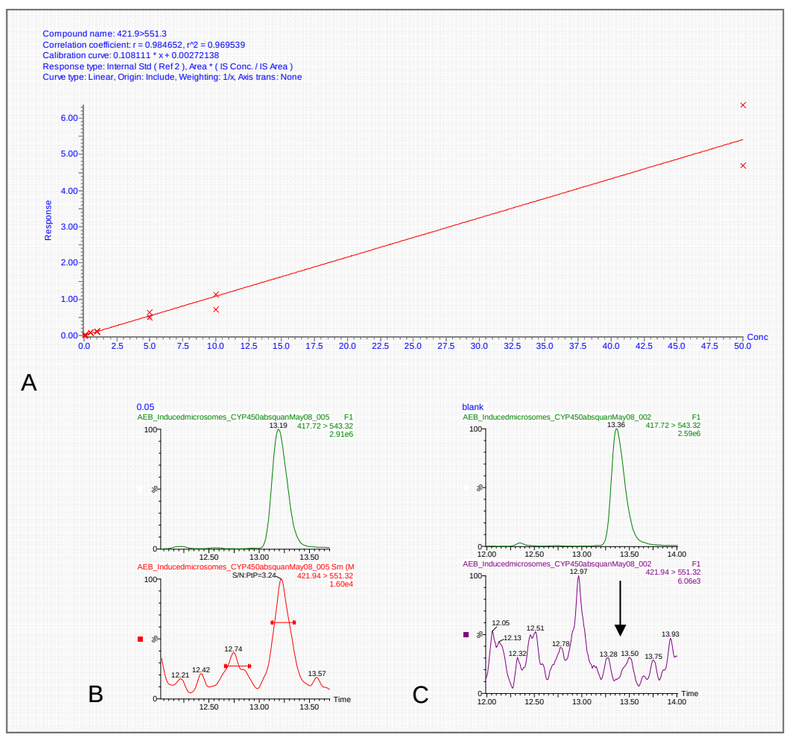


Figure 4. MRM analysis on Quattro Premier XE.

A: Example calibration line 0.05-50 fm/µL 2C6 labelled peptide 421.9>551.3 , r^2 = 0.97.

B: LLOQ for transition 421.9>551.3 with endogenous matrix peptide peak 417.1> 543.3.

C: Blank for transition 421.9>551.3 with endogenous matrix peptide peak 417.1> 543.3.

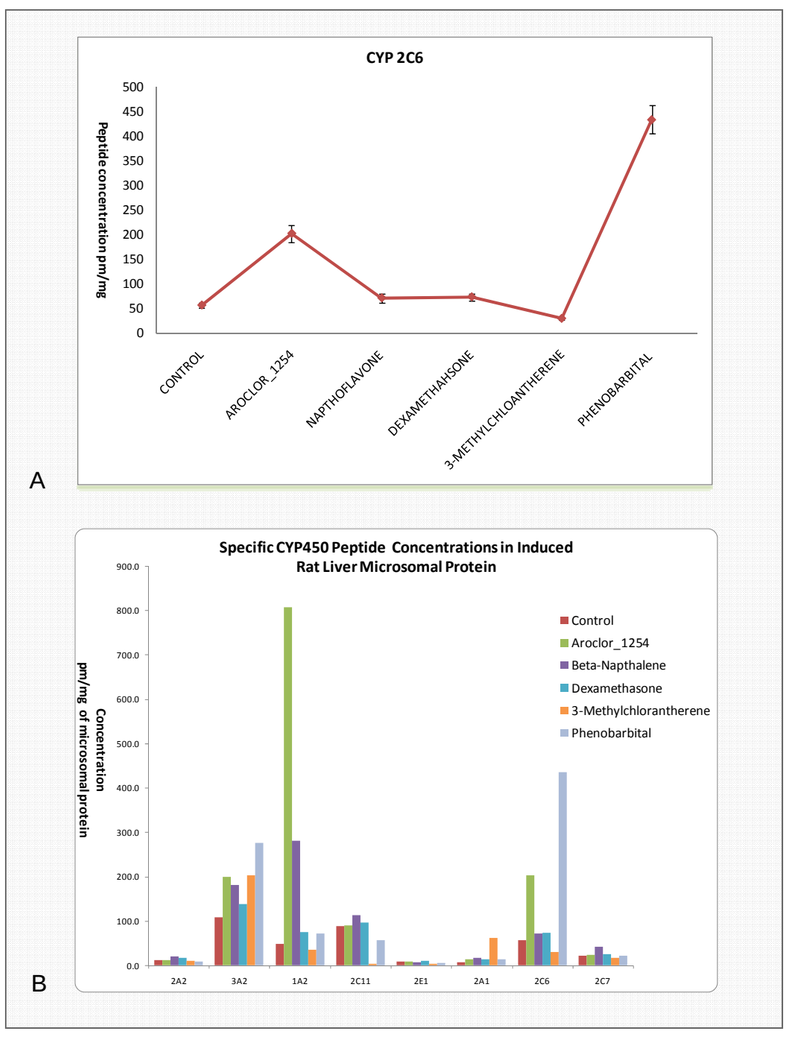


Figure 5. CYP 450 Peptide MRM quantitation by Quattro Premier XE.

A. Variation in absolute concentration of peptide GSFPVAEK (MRM 417.7>543.3) in induced microsomes . This peptide is proteotypic to CYP2C6 enzyme.

B. Absolute concentration of CYP450 enzymes in pm per mg of in rat liver microsome protein. The variation in concentration in induced microsome samples is displayed.

CONCLUSIONS

- **LC-MS^E data was used as a discovery proteomics system to identify proteins in the complex liver microsome biological sample**
- **Automated data sorting tools applied to proteomic discovery data identified precursor and fragment ion information for proteotypic peptides. This data can be used to build triple quadrupole MRM methods**
- **The MRM transitions were used to quantify CYP450 proteins from their proteotypic peptides against their labelled analogues using the method of stable isotope dilution**
- **Limit of quantification of the labelled (and therefore endogenous) peptides in liver microsome digest was between the range of 0.05 and 0.5fm/µL with r^2> 0.96 for all calibration curves to 50fm/µL. %CV of triplicate injections was within the acceptable for quantification (< 17%)**
- **Increases and decreases in CyP450 levels related to chemically induced perturbation of the enzymes could be monitored using this approach**
- **Using the above workflow, automated MRM method building can be achieved**

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