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

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INTRODUCTION

The development of a biomarker assay typically involves the detection, identification and quantitation of multiple proteotypic peptides. Given that, in any one biomarker study, there may be numerous samples containing vast numbers of proteins, a holistic approach to identification and subsequent quantitation 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.

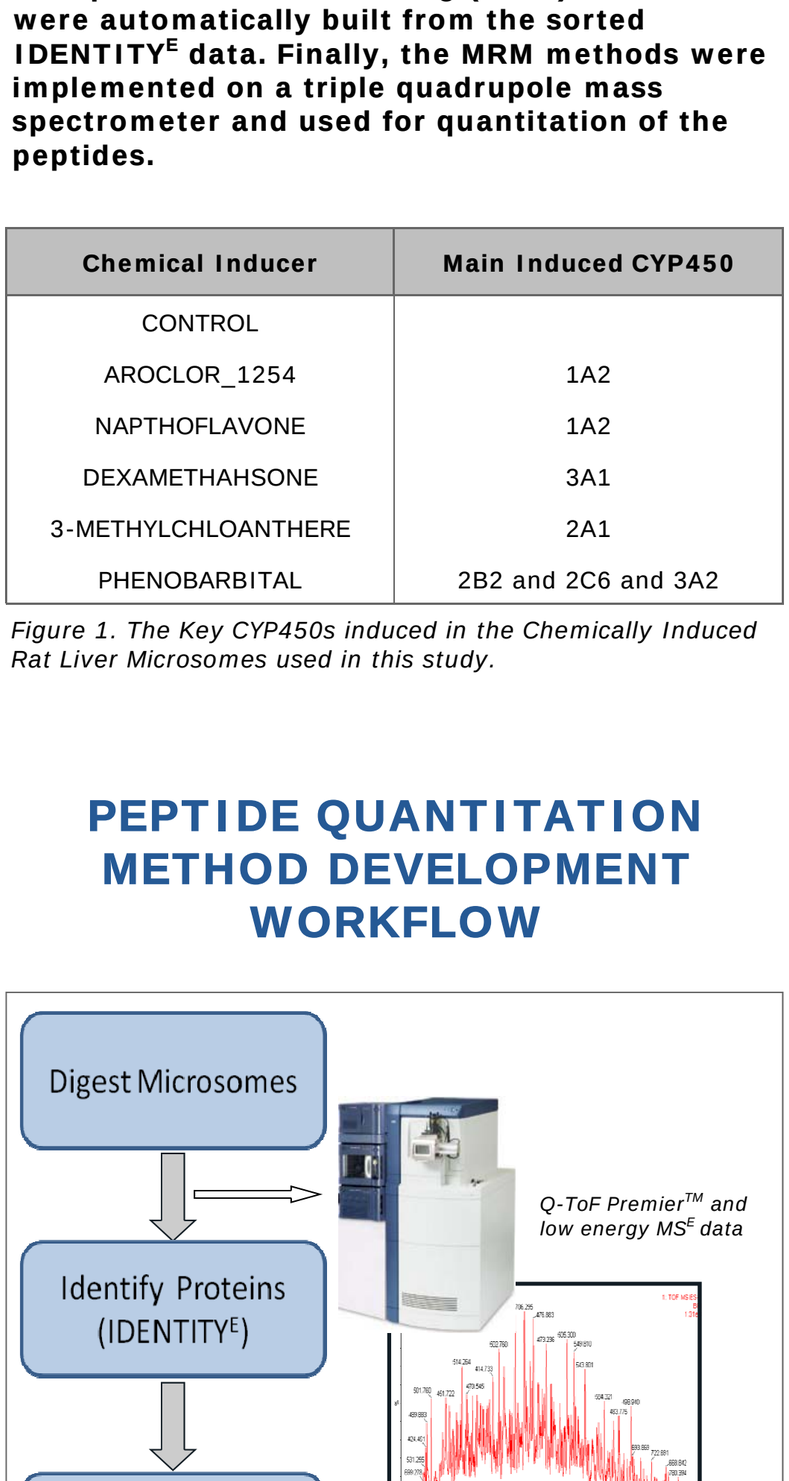
This study describes the complete workflow for development of a biomarker assay, from protein identification through to peptide quantification. Here the workflow is applied to identification and quantitation of key CYP450 enzymes in control and chemically induced rat microsomes (liver cells), the latter of which should display up or down regulation of the CYP450 enzymes (see figure 1).

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

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 QUANTITATION 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 1/20 in 0.1% formic acid containing 10fm/µl ADH digest (Waters MassPREP™ digest standards). Samples were prepared in triplicate to assess reproducibility.

Standards

ADH digest was serially diluted and spiked into a 1/20 dilution of control microsomal protein digest of to give a final calibration range of 1-100fm/µL. Quality control standards were prepared in triplicate at 5 and 10 fm/µl of ADH.

DATA ACQUISITION AND PROCESSING

MASS SPECTROMETRY

Waters® IDENTITY^E system; Waters Q-Tof Premier 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 using IDENTITY^E processing.

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 Premier. Two MRM transitions were acquired for each proteotypic peptide (one or two 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 15).

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). Peak elution occurred 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.

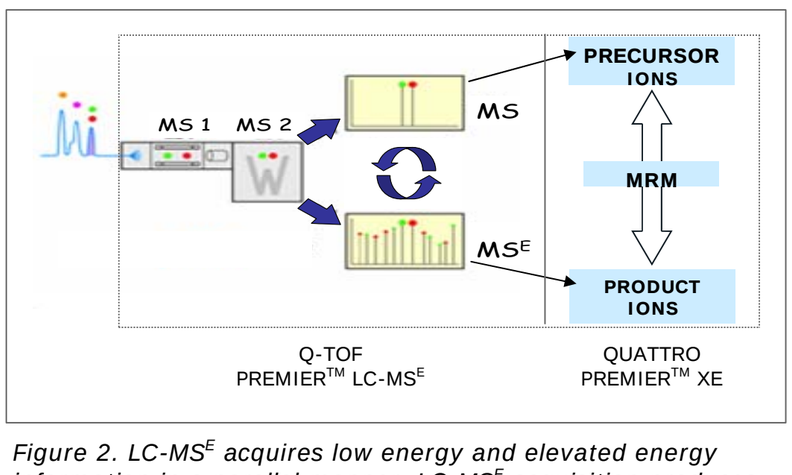


Figure 2. LC-MS^E acquires low energy and elevated energy information in a parallel manner. LC-MS^E acquisition produces an inventory of all detectable precursor and fragment ions through the entire chromatographic run, along with their corresponding intensities . This information is sorted to obtain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

RESULTS

Func	prec.mz	prod.mz	RT	Protein.Num	Transition.Num	Cone V	Collision E	pep.seq		Protein.Transition.Number	CyP450
1	725.9	923.5	72.2	11196	5	30	28.0	AVEDLNNLJFFR	-	11196-5	4A2
1	725.9	469.3	72.3	11196	4	30	28.0	AVEDLNNLJFFR	-	11196-4	4A2
1	552.8	545.3	44.0	11196	2	30	22.1	LYSPVPSVSR	-	11196-2	4A2
1	552.8	741.4	44.0	11196	1	30	22.1	LYSPVPSVSR	-	11196-1	4A2
1	620.4	928.5	55.2	10958	1	30	24.4	IVVLYGYDAVK	-	10958-1	2A2
1	620.4	1027.5	55.2	10958	2	30	24.4	IVVLYGYDAVK	-	10958-2	2A2
1	411.3	460.3	50.1	10958	1	30	17.3	FTIATLR	-	10958-1	2A2
1	411.3	573.4	50.1	10958	2	30	17.3	FTIATLR	-	10958-2	2A2
1	613.3	1027.5	52.8	10950	2	30	24.2	VVVLYGYDAVK	-	10950-2	2A1
1	613.3	928.5	52.8	10950	4	30	24.2	VVVLYGYDAVK	-	10950-4	2A1
1	754.4	894.5	72.2	10950	1	30	29.0	TVSNVISSIVFGER	-	10950-1	2A1
1	754.4	1007.6	72.2	10950	2	30	29.0	TVSNVISSIVFGER	-	10950-2	2A1
1	711.3	1009.5	60.8	11106	1	30	27.5	EALVDLGEEFSGR	-	11106-1	2C11
1	711.3	781.3	60.8	11106	2	30	27.5	EALVDLGEEFSGR	-	11106-2	2C11
1	451.7	675.3	26.2	11106	1	30	18.7	VQEEIER	-	11106-1	2C11
1	451.7	786.4	26.2	11106	2	30	18.7	VQEEIER	-	11106-2	2C11
1	650.3	1058.5	43.0	11187	1	30	25.4	LQEEIDGALPSK	-	11187-1	3A2
1	650.3	800.5	43.0	11187	4	30	25.4	LQEEIDGALPSK	-	11187-4	3A2
1	630.3	1031.6	70.8	11187	3	30	24.7	DIELDGLFIPK	-	11187-3	3A2
1	630.3	789.5	70.8	11187	4	30	24.7	DIELDGLFIPK	-	11187-4	3A2
1	418.3	601.4	60.5	10968	3	30	17.5	FSLSILR	-	10968-1	2E1
1	418.3	488.3	60.5	10968	2	30	17.5	FSLSILR	-	10968-2	2E1
1	603.3	617.4	57.0	10968	1	30	23.8	EAQFLVEELK	-	10968-1	2E1
1	603.3	730.4	57.0	10968	2	30	23.8	EAQFLVEELK	-	10968-2	2E1
1	584.8	964.5	56.6	10910	2	30	23.2	GFGIVFSNGNR	-	10910-2	2C7
1	584.8	907.5	56.6	10910	5	30	23.2	GFGIVFSNGNR	-	10910-5	2C7
1	417.7	543.3	34.0	10915	1	30	17.5	GSFPVAEK	-	10915-1	2C6
1	417.7	446.3	34.0	10915	3	30	17.5	GSFPVAEK	-	10915-3	2C6
1	1063.5	893.5	78.9	11333	4	30	39.5	DFVENVTSGNAVDFFPVLR	-	11333-4	1A2
1	1063.5	1422.7	78.9	11333	5	30	39.5	DFVENVTSGNAVDFFPVLR	-	11333-5	1A2
1	653.9	992.5	68.3	11333	1	30	25.5	NSIQDITGALFK	-	11333-1	1A2
1	653.9	864.5	68.3	11333	6	30	25.5	NSIQDITGALFK	-	11333-6	1A2

Figure 3. Summary of the ion inventory from the Identity data for the CYP450 proteins, after filtering using a series of criteria. Final proteotypic peptides (precursor and fragment ions) are displayed. MRM transitions for triple quadrupole analysis were automatically built from data in the above table. (Collision energy calculation; ce= 0.034m/z + 3.314)

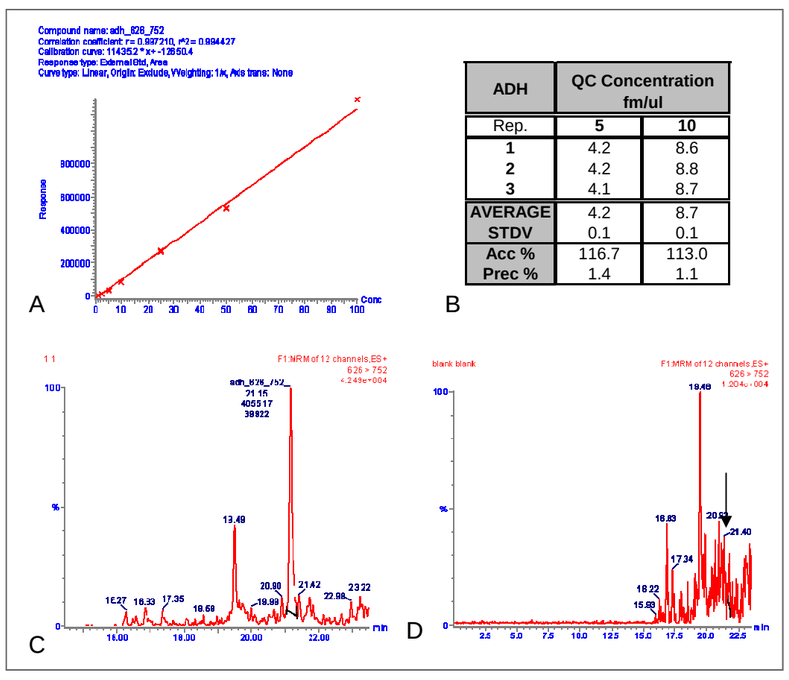


Figure 4. MRM analysis on QuattroPremier XE.

A: ADH Duplicate Calibration Line 1-100fm/ul , r = 0.997,
B: QC accuracy and Precision data
C: 1fm/ul LOQ,
D: Blank

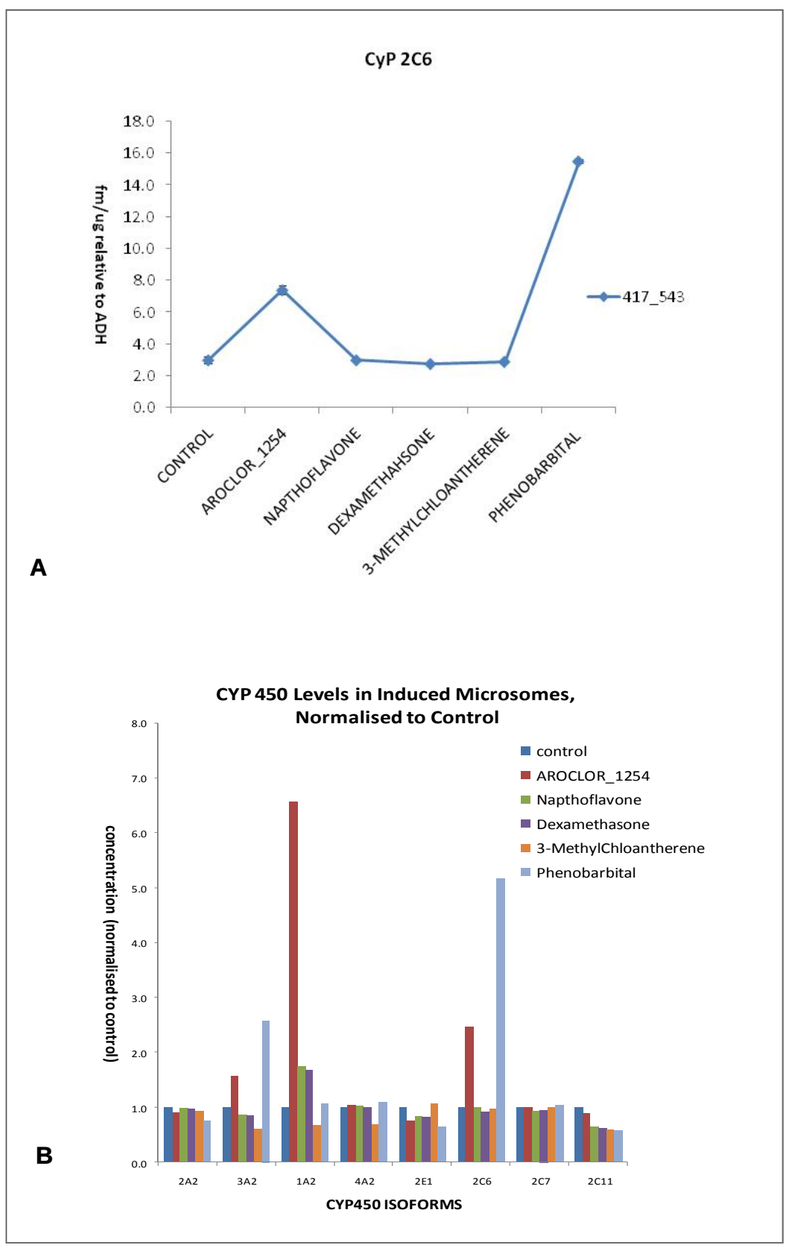


Figure 5. CYP 450 Peptide MRM quantitation by Quattro Premier XE.

1. *Variation in levels (rel. to ADH) of peptide GSFPVAEK (MRM 417.7>543.3). This peptide is proteotypic to CYP 2C6 enzyme.*
2. *Normalised levels of CYP450 enzymes displaying variation in induced microsome samples.*

CONCLUSIONS

- **IDENTITY^E using LC-MS^E data acquisition can be used to identify proteins in a complex biological matrix such as liver microsome digest**
- **Automated data sorting tools can be applied to IDENTITY^E data to identify 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 (relative to ADH) CYP450 proteins from their proteotypic peptides, in control and induced Rat Liver Microsomes, using a triple quadrupole mass spectrometer**
- **Limit of quantitation of ADH in liver microsome digest was 1fm/µL and a linear calibration range to 100fm/uL observed. QC accuracy and precision data was within limits usually acceptable in a typical small molecule quantitation assay.**
- **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 compliment the specificity advantages offered by IDENTITY^E peptide identification and the sensitivity advantages offered by triple quadrupole mass spectrometry for peptide quantitation**

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