4-Plex iTRAQ Based Quantitative Proteomic Analysis Using an Agilent Accurate-Mass Q-TOF

Application Note

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Abstract

Differentially expressed proteins in tuberculous meningitis were identified using the Agilent 6520 Accurate-Mass Q-TOF LC/MS System. The excellent resolution and dynamic range achieved in the LC/MS/MS analysis enabled quantitation and identification of eight hundred proteins from the iTRAQ (Isobaric Tags for Relative and Absolute Quantitation) labeled brain tissue samples. Interferences in the iTRAQ reporter ion region were minimized due to the high resolution achieved in the low mass (*m/z*) region.

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Introduction

Quantitative proteomic studies measure the relative amounts of proteins in a biological system of interest in order to determine how those protein levels vary in response to a particular condition such as disease progression. The iTRAQ quantitative proteomics approach has emerged as a powerful method for protein expression profiling studies, due to its ability to identify and quantitate proteins simultaneously.[1] In this study, iTRAQ was used in conjunction with LC/MS to identify differentially expressed proteins during tuberculous meningitis, a clinical condition caused by *Mycobacterium tuberculosis* infection.

The resolving power of the mass spectrometer is a key factor for separating the iTRAQ reporter ions from one another and from other contaminants in the low mass region, since the reporter ions have *m/z* values of 114, 115, 116, and 117 in a 4-plex iTRAQ experiment. High resolution is essential for reliable quantitation of the reporter ion intensities, while acquiring data at a higher acquisition rate, in order to maximize protein identification. This application note describes the use of the Agilent 6520 Accurate-Mass Q-TOF LC/MS System coupled with the 1200 Series HPLC-Chip/MS System and Spectrum Mill MS Proteomics Workbench data analysis suite to identify 800 proteins in brain tissue samples. Of these, 250 were found to be differentially expressed between the infected and non-infected samples by a factor of two-fold or more.

Experimental

Sample preparation

Proteins extracted from six pathologically proven autopsy brain tissue (frontal cortex) samples of tuberculous meningitis (TBM) were pooled and 80 µg of protein was used for the study. Proteins extracted from six normal brain samples (age-sex matched) of head injury cases were pooled and 80 µg of protein was used as the control sample.

iTRAQ labeling

Protein digestion using trypsin and iTRAQ labeling of resulting peptides were carried out as per the iTRAQ Reagents Multiplex Kit protocol. Peptides obtained from normal brain samples (control) were labeled using iTRAQ reagents containing 114 and 115 reporter ions, while peptides from infected brain tissue samples were labeled with 116 and 117 reporter ions.

Fractionation by strong cation exchange chromatography

After tryptic digestion and iTRAQ labeling, the labeled peptides were fractionated using strong cation exchange chromatography. The chromatography was carried out using an Agilent 1200 Series HPLC system on a 200 x 2.1 mm PolySULFOETHYL A (5 µm; 200 Å) column. Solvent A was 20% acetonitrile in 10 mM KH_2PO_4 (pH 2.7) and solvent B was 20% acetonitrile in 10 mM KH_2PO_4 mixed with 350 mM KCl (pH 2.7). The following gradient was used for elution: 0% B until 20 min; 8% B at 22 min; 50% B at 52 min; 100% B at 65-70 min; and 0% B at 70.5-90 min. A total of 90 fractions were collected, and 30 fractions were selected for further analysis, based on the UV absorbance at 214 nM. Selected fractions were concentrated to reduce the sample volume.

LC/MS/MS analysis

An Agilent 6520 Accurate-Mass Q-TOF LC/MS System equipped with a 1200 Series HPLC-Chip/MS System.

HPLC-Chip conditions

Chip and columns: HPLC-Chip with a 75 µm x 150 mm analytical column packed with ZORBAX 300SB C18 (5 µm; 300 Å), and a 160 nL enrichment column.

Injection volume: 8 µL

Flow: 300 nL/min analytical pump, 4 µL/min loading pump.

Mobile phases: A: 0.1% formic acid in water; B: 90% acetonitrile in water with 0.1% formic acid.

Gradient: 3% B at 0 min; 18% B at 4 min; 40% B at 45 min; 90% B at 55-60 min, then 3% B at 62 min.

Stop time: 70 min

Accurate-Mass Q-TOF LC/MS conditions

Mode: positive ion

Acquisition parameters: MS mode: 1 spectrum/sec, *m/z* 350-1800; MS/MS mode: 3 spectra/sec, *m/z* 60-2000.

Drying gas: 5 L/min, 325°C

Collision energy: slope 3, intercept 2

Capillary voltage: 1900 V

Data analysis

Spectrum Mill software was used for protein identification and quantitation of iTRAQ reporter ion intensities. Identified proteins were validated using the criteria of a minimum peptide score of 6 and a protein score of 10 in the Spectrum Mill software. MassHunter Qualitative Analysis software was used to view the spectra.

Results and Discussion

Eight hundred proteins were identified from the LC/MS/MS analysis of 30 fractions collected from the cation exchange fractionation. A partial list (top 18 proteins) of identified and validated proteins is shown in **Figure 1**. Intensities of the reporter ions at *m/z* 114, 115, 116, and 117 were compared using the Spectrum Mill software suite to determine differential expression of proteins. Proteins were considered differentially expressed if the reporter ion intensities differed between the normal and infected samples by 2-fold or more. The technical variability was estimated using the ratio of reporter ions 114 and 115 in the uninfected samples. The coefficient of variation (CV) calculated from the reporter ion ratios (115/114) of the top 20 proteins identified in this study was 29%.

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Species	Database Accession #	Protein Name
1	34	12	153.17	39	Homo sapiens	21361322	tubulin, beta 4
2	35	9	132.49	5	Homo sapiens	4507191	spectrin, alpha, non-erythrocytic 1 (alpha-fodrin)
3	18	10	119.06	6	Homo sapiens	112382250	spectrin, beta, non-erythrocytic 1 isoform 1
4	44	6	90.26	23	Homo sapiens	57013276	tubulin, alpha, ubiquitous
5	33	5	86.28	20	Homo sapiens	4885063	fructose-bisphosphate aldolase C
6	23	7	85.93	31	Homo sapiens	5803011	enolase 2
7	17	7	85.77	5	Homo sapiens	4758012	clathrin heavy chain 1
8	25	6	82.10	17	Homo sapiens	32189394	ATP synthase, H+ transporting, mitochondrial F1 complex, beta subunit precursor
9	20	7	81.05	18	Homo sapiens	4757810	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit precursor
10	26	6	79.28	15	Homo sapiens	4503377	dihydropyrimidinase-like 2
11	37	5	75.21	19	Homo sapiens	4501887	actin, gamma 1 propeptide
12	9	6	68.41	11	Homo sapiens	105990539	neurofilament, light polypeptide 68kDa
13	52	4	67.31	13	Homo sapiens	21536286	brain creatine kinase
14	13	5	61.38	26	Homo sapiens	34577110	aldolase A
15	11	4	57.68	9	Homo sapiens	33286420	pyruvate kinase 3 isoform 2
16	9	4	57.00	5	Homo sapiens	59853099	dynamin 1 isoform 1
17	16	5	56.04	26	Homo sapiens	7669492	glyceraldehyde-3-phosphate dehydrogenase
18	7	4	53.05	12	Homo sapiens	4503979	glial fibrillary acidic protein

Figure 1. Example table of results from a database search using Spectrum Mill.

Figure 2 shows an expansion of the *m/z* region 114-118 from the MS/MS spectrum of the peptide CSTILLQGK, derived from the protein Na/K+-ATPase alpha3 subunit. It illustrates the excellent resolution achieved in this *m/z* region (~10000). The reporter ions are clearly well resolved, enabling reliable quantitation based on their intensities. Ratios of reporter ion intensities from four peptides derived from the protein Na/K+-ATPase alpha3 subunit are listed in **Table 1**. The ratios for infected versus normal tissue (116/114 and 117/114) are less than 2-fold in all four peptides, indicating that there is no significant change in expression of this protein between the normal and infected samples.

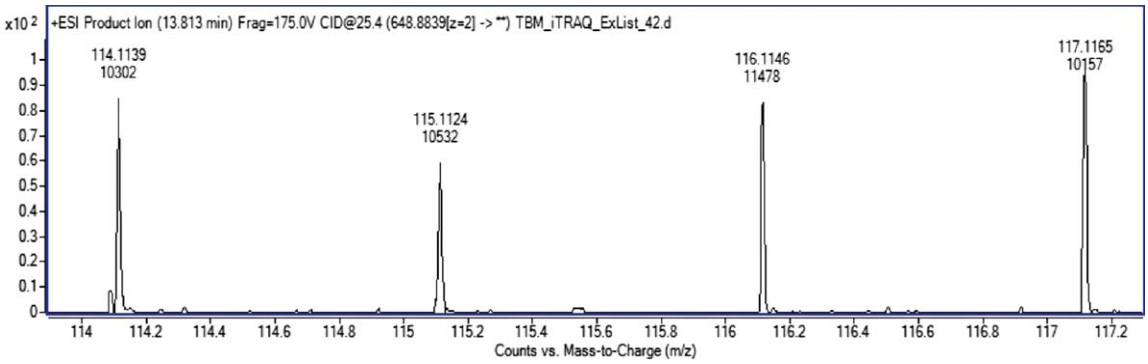

Figure 2. The m/z region 114-118 in the MS/MS spectrum of the peptide CSTILLQGK from the protein Na/K+-ATPase alpha3 subunit.

Peptide sequence	Peptide score	115/114	116/114	117/114
DVAGDASESALLK	21.3	0.8	1.4	1.2
GGQDNIPVLK	14.3	0.7	1.3	1.2
LNIPVSQVNPR	14.2	0.9	1.4	1.4
CSTILLQGK	11.0	0.7	1.0	1.3

Table 1. Intensity ratios of the reporter ions in four peptides from the protein Na/K+-ATPase alpha3 subunit.

As expected, expression levels of most of the proteins did not change between the infected and normal samples. Among the total 800 proteins identified in this study, 250 proteins were found to be two-fold or more differentially expressed in tuberculous meningitis infected brain as compared to uninfected brain. The differentially expressed proteins included glial fibrillary acidic protein[2] (GFAP) and lactate dehydrogenase A[3] (LDHA), which were previously reported to be involved in tuberculous meningitis. Several novel proteins like N-myc downstream regulated 1 (NDRG1), vacuolar protein sorting 35 (VPS35) and stress-induced phosphoprotein 1 (STIP1), which have not previously been reported to be involved in tuberculous meningitis infection, were also identified. **Figure 3** shows the MS/MS spectrum of the peptide FADLTDAAAR from glial fibrillary acidic protein, and **Figure 4** shows the reporter ion intensities from this peptide, which were almost three-fold higher in the infected sample as compared to normal sample. Intensity ratios from this as well as three other peptides from the same protein are listed in **Table 2**, confirming that there was more than two-fold up-regulation of the protein in the infected sample. Glial fibrillary acidic protein is an astrocyte marker, which is known to be involved in astrocyte injury and damage in the Alzheimer's disease brain.

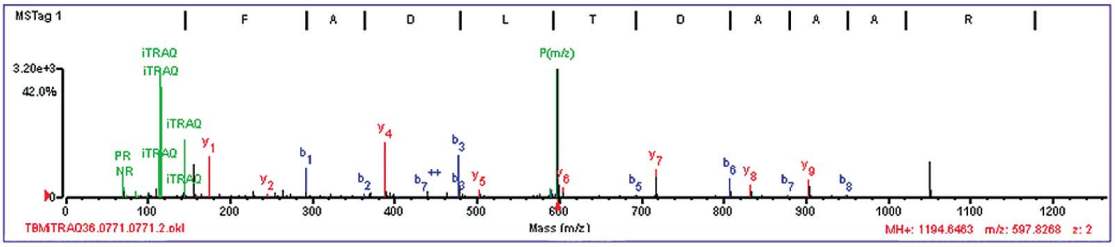

Figure 3. MS/MS spectrum of the peptide FADLTDAAAR from glial fibrillary acidic protein.

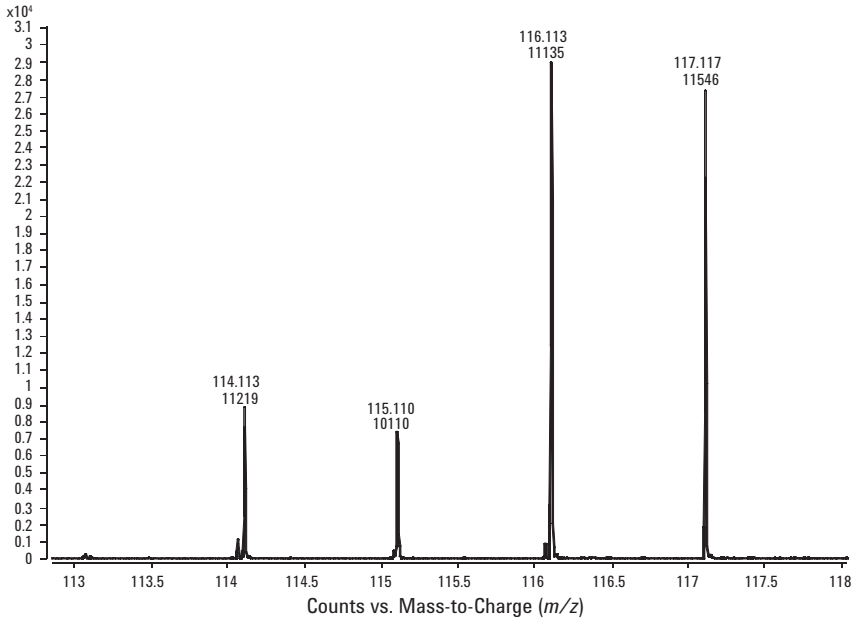

Figure 4. Expansion of the m/z region 113-117 in the MS/MS spectrum of the peptide FDLTDAAAR from glial fibrillary acidic protein.

Peptide sequence	Peptide score	115/114	116/114	117/114
LADVYQAELR	18.4	1.0	2.6	2.8
LEAENNLAAYR	17.4	0.8	2.3	2.2
DNLAQDLATVR	17.3	0.8	2.7	2.6
FADLTDAAAR	16.2	0.9	2.9	2.7

Table 2. Intensity ratios of the reporter ions in four peptides from glial fibrillary acidic protein.

Conclusion

The Agilent 6520 Accurate-Mass Q-TOF LC/MS System coupled with the 1200 Series HPLC-Chip/MS System is a powerful, high resolution platform for iTRAQ proteomic analysis. Reporter ions from iTRAQ labeled peptides can be well resolved without interference from one another or from other contaminants.

A total of 800 proteins were identified from brain tissues, 250 of which were differentially expressed in tuberculous meningitis. The list of differentially expressed proteins included several novel proteins like vacuolar protein sorting 35 and stress-induced phosphoprotein, which have not previously been reported in tuberculous meningitis infection. These results confirm the utility of this system for identifying potential biomarkers that may be useful for early diagnosis.

References

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Published in the U.S.A., June 10, 2010
5990-5881EN

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