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Determination of Lys-40 Acetylated Alpha-Tubulin in Rat Brain Tissue by Immuno Precipitation-Mass Spectrometry (IP-MS)

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Abstract

Acetylation at α-tubulin Lys-40 is a key post-translational modification (PTM) in the nervous system and is a potential biomarker for neurodegenerative diseases and xenobiotic-induced neurotoxicity. However, the absolute level of this key PTM has not been determined in the brain. Immunoprecipitation-mass spectrometry (IP-MS) combines antibody specificity and mass spectrometry selectivity and is being widely applied for the targeted quantitation of proteins within complex biomatrices. Lys-40 acetylation has not been previously studied with MS due to the lack of cleavage sites in the vicinity for trypsin and other common enzymes, making it challenging to identify a surrogate peptide for MS analysis. In this study, we report that such a peptide was identified for the first time after pepsin digestion, and an IP-MS-based assay was developed to absolutely quantitate Lys-40 acetylated α-tubulin in rat brain tissue. The workflow includes pepsin digestion, immunoaffinity enrichment of the acetylated peptide, and quantitation using a stable isotope-labelled peptide. Only a small amount of brain tissue (2 mg) was required for each analysis. The method provided a linear range of 1.0–100.0 pmol/mg brain tissue, and selectivity, precision, and accuracy were validated. This method has been successfully applied in a preclinical study of chlorpyrifos neurotoxicity, and we observed a significant decrease of brain tubulin acetylation after chronic exposure to this organophosphate insecticide.

Keywords: Tubulin acetylation, immunoprecipitation, mass spectrometry, chlorpyrifos and neurotoxicity

Introduction

Tubulin is a protein superfamily including α-, β-, γ-, Δ-, δ-, ε- and ζ-tubulins [1]. Heterodimers of α- and β-tubulin polymerize into microtubules accompanying GTP hydrolysis. Microtubules alternate between periods of growth and shrinkage known as “dynamic instability” [2]. This property is essential for cellular activities including cell migration, division, and development.

In neurons, microtubules serve as “railways” of the axonal transport system, responsible for the transport of organelles, proteins, mRNA, and signaling molecules by motor proteins. Kinesin delivers cargo from the cell body to the synaptic terminal, and dynein delivers cargo in the opposite direction.

Tubulin receives a host of post-translational modifications (PTMs) in the nervous system, including acetylation [3], detyrosination [4], Δ2 modification [5], polyglutamylation [6], and polyglycylation [7]. These PTMs, except for tubulin acetylation, occur at the C-terminal region of α- or β-tubulin on the outer surface of microtubules [8]. By contrast, tubulin acetylation occurs at Lys-40 on the lumen surface and is preserved in α-tubulin isotypes.

Acetylation is mediated by α-tubulin acetyltransferase 1 (ATAT1/MEC-17) from the

inside out, and its deacetylation is mediated by histone deacetylase 6 (HDAC6) and NAD-dependent deacetylase sirtuin-2 (SIRT2), allowing for a balanced regulation of tubulin acetylation [9,10].

Lys-40 acetylation is associated with dendrite growth, microtubule stabilization, and binding with motor proteins [11]. Experiments have shown that kinesin-1 binds to microtubules only when Lys-40 of α-tubulin is acetylated [11]. Decreases in tubulin acetylation have been observed in multiple neurodegenerative diseases [12,13] and neurotoxicity induced by various toxicants [14,15]. In patients with Huntington's disease (HD), a reduction in tubulin acetylation was concomitant with decreased transport of microtubule-dependent vesicles containing brain-derived neurotrophic factor (BDNF). These transport defects could be rescued by increasing tubulin acetylation using the HDAC inhibitor trichostatin A (TSA). TSA treatment enhanced the recruitment of motor proteins including kinesin-1 and dynein [12]. Similarly, diisopropyl fluorophosphate (DFP), an organophosphate insecticide, was associated with a decline in tubulin acetylation. This alteration, along with deficits in microtubule dynamics, mitochondrial transport, and dopamine release, was found to be correctable or improved by tubacin, a drug that inhibits HDAC6 [14].

Considering tubulin acetylation as a promising biomarker for multiple neuronal deficits, it is necessary to develop analytical methods for this key PTM. Western blot is most commonly used for detecting tubulin acetylation [16,17]. One validated method, using chemically acetylated tubulin as a standard, estimated the percentage of acetylated tubulin relative to total tubulin in three different cell lines (0–7%). However, potential cross-reactivity of the antibody with other proteins or acetylated lysines on tubulin [16,18] can complicate results.

Immunoprecipitation-mass spectrometry (IP-MS) has emerged as an important technique for targeted quantitation of proteins in biological matrices. It reduces matrix complexity through antibody specificity and detects analytes with mass spectrometry selectivity [19]. It typically relies on a surrogate peptide generated via tryptic digestion, but the lack of cleavage sites near Lys-40 complicates generation of such a peptide.

In this study, a suitable peptide was successfully identified after pepsin digestion. We developed and validated the first MS-based assay, using immunoprecipitation for surrogate peptide enrichment and selected ion recording (SIR) mass spectrometry for detection, to absolutely quantitate Lys-40 acetylated α-tubulin in rat brain tissue.

This method was successfully applied in a chronic neurotoxicity study of the organophosphate insecticide chlorpyrifos, contributing to the mechanistic understanding of organophosphate neurotoxicity.

Materials and Methods

Acetyl-α-tubulin (Lys 40)monoclonalantibodies:mousemonoclonalantibody(mAb)[6- 11B-1] was acquired from Novus Biologicals (Littleton, CO) and rabbit mAb [D20G3] was purchased from Cell Signaling Technology (Boston, MA). Protein G magnetic beads were obtainedfromBio-rad (Hercules,CA). Wholeratbrains(WistarHannover)werepurchasedfrom Bioreclamation IVT (Westbury, NY). Pure tubulin (bovine) was purchased from Cytoskeleton (Denver, CO). Modified pepsin (porcine) was purchased from Princeton Separations (Adelphia, NJ). Protease inhibitors trichostatin A (TSA), phenylmethylsulfonyl fluoride (PMSF), 1, 10- phenanthroline were purchased from Cayman Chemical (Ann Arbor, MI), Research Products International (Mt Prospect, IL) and Oakwood Chemical (Estill, SC), respectively. Fmoc amino acids and resins for solid phase peptide synthesis, including Fmoc-Leu-OH ($13C_6$,15N) and Fmoc-Lys(Ac)-OH, were purchased from ChemPep (Wellington, FL). 2-(N- morpholino) ethanesulfonic acid (MES) anhydrous was purchased from Amresco (Solon, OH). Glycine, tris(hydroxymethyl) aminomethane, Ethylenediaminetetraacetic acid (EDTA), magnesium chloride, dithiothreitol (DTT), hydrochloric acid, and LC-MS grade solvents including formic acid, trifluoroacetic acid, acetonitrile and water were purchased from Sigma−Aldrich (St. Louis, MO).

Solid Phase peptide synthesis

Peptides YCLEHGIQPDGQMPSDKTIGGGDDSF (Lys-40 peptide), YCLEHGIQPDGQMPSDK(ac)TIGGGDDSF (surrogate peptide for Lys-40 acetylated α-tubulin), and YCL*EHGIQPDGQMPSDK(ac)TIGGGDDSF (L* = 7 Da mass shift, stable isotope labelled peptide, SIL peptide) were synthesized using Fmoc solid-phase peptide synthesis with the AAPPTec Focus XC automated peptide synthesizer (Louisville, KY). Peptides were purified with an Agilent Polaris 5C18-A preparative column (150 × 21.2 mm, 5 μm) on the Shimadzu LC-20AT HPLC system with a SPD-20A UV-Vis detector (Kyoto, JP).

LC-MS/MSconditions

Mobile phase A was water containing 0.01% (v/v) formic acid, and mobile phase B was acetonitrile. A Halo® peptide ES-C18 column (100 × 4.6 mm, 2.7 μm) by Advanced Materials Technology (Wilmington, DE) coupled with a C-18 guard column (4.0 mm ×2.0 mm) by Phenomenex (Torrance, CA) was used to separate the peptides. The injection volumewas20μL. Different LC-MS instrumentation and parameters were applied for peptide sequencing and quantitation. For peptide sequencing, A Waters UPLC system (Milford, MA) coupled to a Waters SYNAPT G2 Q-TOF mass spectrometry with an ESI source (Milford, MA) was operated for LC−MS/MS analysis. Peptides were separated using a gradient method, with a 0.3 mL/min flow rate, (minute, % mobile phase B): (0, 5), (60, 50), (60.01, 95), (67.50, 95), and the run time for each injection was 75 min. MS setting for the SYNAPT G2 mass spectrometer were: capillary voltage 2.00 kV, sample cone voltage: 35 V, extraction cone voltage 4.0 V, source temperature 120 °C, desolvation temperature 500 °C and desolvation gas 500 L/h. Data were acquired with the data-dependent acquisition (DDA) function: 1s MS survey scan in the mass range of 300–1900 were followed by MS/MS scans of up to 3 ions, when intensity rose above 1500 counts/s. MS/MS scan was acquired over the range of 100-1900, with a 2 s scan rate, and was switched to MS survey scan after 3 scans. Trap collision energy was set using charge state recognition, applying the default files for 1-4 charge states. For peptide quantitation, an Agilent 1100 binary pump HPLC system (Santa Clara, CA) interfaced to a Waters Micromass Quattro A Micro triple quadrupole mass spectrometer with an ESI source (Milford, MA) was operated for LC−MS analysis. The capillary voltage was 3.80 kV, the cone voltage was 32 V, the extractor voltage was 1, and the RF lens voltage was 2.0 V. The source temperature was 120 °C, the desolvation gas flow rate was 500 L/h, and the desolvation temperature was 500 °C.

Peptides were separated using a gradient method, with a 0.3 mL/min flow rate, as follows (minute, % mobile phase B): (0, 25), (15, 40), (15.01, 95), (22.50, 95). The run time for each injection was 30 minutes.

The Selected Ion Recording (SIR) function was applied to monitor precursor ions m/z 1406.0 and 1409.5 at the retention time of 7.5 min for the surrogate peptide and the SIL peptide, respectively.

Data were processed with Waters software, including ProteinLynx Global Server (PLGS) 2.4 and MassLynx 4.1 (Beverly, MA).

Tissue Extract

One volume of rat brain tissue was resuspended in 1.5 volumes of MES buffer (100 mM MES, 1 mM magnesium chloride, 1 mM EDTA, pH 6.8) supplemented with 1 mM PMSF, 1 mM 1,10-phenanthroline, and 5 μM of the HDAC inhibitor trichostatin A (TSA).

Brain homogenate was prepared on ice using a tissue grinder. The homogenate was frozen at −80 °C for 30 minutes and thawed at 37 °C. The homogenate was sonicated 5 times for 10 seconds with 30-second rest intervals on melted ice.

The sample was centrifuged at 30,000 × g (Beckman TL 120.2) at 4 °C for 15 minutes to remove cell debris, and the supernatant was then centrifuged at 100,000 × g (Beckman TL 120.2) at 4 °C for 1 hour. The final supernatant was collected as brain tissue lysate.

Protein concentration was determined using a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

Pepsin Digestion and Immunoprecipitation of the acetylated peptide

Brain tissue lysate was mixed with urea to a concentration of 2 M and acidified to pH 2 with hydrochloric acid. Proteins were digested with pepsin (enzyme: total protein = 1:2000) at 37 °C for 18 hours.

Each 5 µL of peptic digest was neutralized and diluted with 45 µL of phosphate-buffered saline supplemented with 0.1% Tween 20 (PBS-T). 1 µL of antibody and 10 pmol SIL peptide were mixed with diluted brain tissue lysate and incubated overnight while rotating at 4 °C.

100 µL of the suspension of protein G magnetic beads were washed with 500 µL of PBS-T three times before incubation with the antibody-antigen mixture for 2 hours while rotating at 4 °C.

The magnetic beads were washed with ice-cold buffers: PBST twice, PBS once, and lastly with 50 mM ammonium acetate (pH 7). Enriched peptides were eluted with 25 µL of 100 mM glycine-TFA (pH 2.0) at room temperature.

Method Validation

The same protocol for the preparation of brain tissue lysate was used to prepare a blank matrix for acetylated tubulin, except that TSA, an HDAC6 inhibitor was absent. Selectivity, linearity, precision, and accuracy tests were conducted to validate the method. Each test was conducted with 50 µL of 10-fold diluted brain tissue lysate, equal to 2 mg of brain tissue. Selectivity (n = 3) was tested by comparing the chromatograms of analytes in blank brain tissue lysate, with analytes in the same matrix at 1 pmol/mg brain tissue. Linearity was validated with calibration standard samples over the range of 1.0–100 pmol/mg. Calibration curves were made from peak area ratios between analytes and the SIL peptide. The specificity and accurary (n=3) were tested by QC samples at low (3 pmol/mg), middle (30 pmol/mg) and high (75 pmol/mg) concentrations.

Animal study

Wistar Hannover rats were orally dosed with 18 mg/kg chlorpyrifos in peanut oil every other dayfor 30 days with a 45 daywashout period. Vehicle was peanut oil with 3% DMSO. All proceduresemployedinthisstudywerereviewedan dapprovedbytheAugustaUniversity Institutional Animal Care and Use Committee and are consistent with AAALAC guidelines. Measures were taken to minimize pain and discomfort in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996. Rats were anesthetized with isoflurane. Whole brains were taken after the washout period, washed in PBS, and kept frozen at −80 °C until analysis.

Results and discussion

Identification of the surrogate peptide

Trypsin is the most commonly used digestive enzyme in proteomics studies, known for its specificity, reproducibility, and efficiency. However, in the vicinity of Lys-40, there is only Arg-2 and Lys-60, and a very long peptide would be generated by trypsin in the presence of acetylated Lys-40, beyond the fragmentation and sequencing capability of common mass spectrometry instrumentation.

Various other enzymes and cleavage reagents, including formic acid, proteinase K, endoproteinase Asp-N, and pepsin, were tested to digest purified tubulin (bovine) to identify a peptide sequence containing Lys-40. After pepsin digestion, a peptide sequence Y24CLEHGIQPDGQMPSDKTIGGGDDSF49 was identified, and a surrogate peptide for acetylated tubulin Y24CLEHGIQPDGQMPSDK(ac)TIGGGDDS F49 was identified after immunoprecipitation enrichment (Figure 1).

This sequence is conserved in all α-tubulin chains expressed in human, rat, and bovine brain, including α1A-, α1B-, α1C-, and α4A-tubulin [20], but not in α8-tubulin, whose sequence is divergent and lacks Lys-40.

In this study, the advantages of pepsin over other enzymes include:

1. Pepsin is an aspartic acid protease, and its digestive activity is not affected by common protease inhibitors supplemented in the lysis

buffer, including serine protease inhibitors, cysteine protease inhibitors, and metalloprotease inhibitors.

2. The sequence contains Cys and Met, which are prone to oxidation, and the oxidation is accelerated at an alkaline pH, therefore the acidic pH of the peptic digestion prevents the degradation of the peptide; 3) Pepsin demonstrated a high efficiency and reproducibility for the generation of a peptide containing Lys-40. Assuming the ratio of α-tubulin to β-tubulin is 1:1 in purified bovine tubulin, and α8-tubulin and other tubulin isotypes are negligible in the brain[21], pepsin digest efficiency was calculated with ratios of peak areas for the Lys-40 peptide, between the synthetic source and the pepsin digest of 1, 10 and 100 µg/mL total bovine tubulin diluted with 1 mg/mL lysozyme. The digestion efficiency was close to 50% across all tested concentrations (Table 1).

m/z	Charge	Peak mW	Peptide mw	Delta (Da)	Delta (ppm)	Log likelihood	Sequence	Modification	RT (min)
923.131	3	2766.369	2766.190	0.179	64.870	52.315	Y24CLEHGIQPDGQMPSDKTIGGGDDSF49		26.93
1405.213	2	2808.410	2808.200	0.210	74.680	28.776	Y24CLEHGIQPDGQMPSDKTIGGGDDSF49	Acetyl K40	28.80

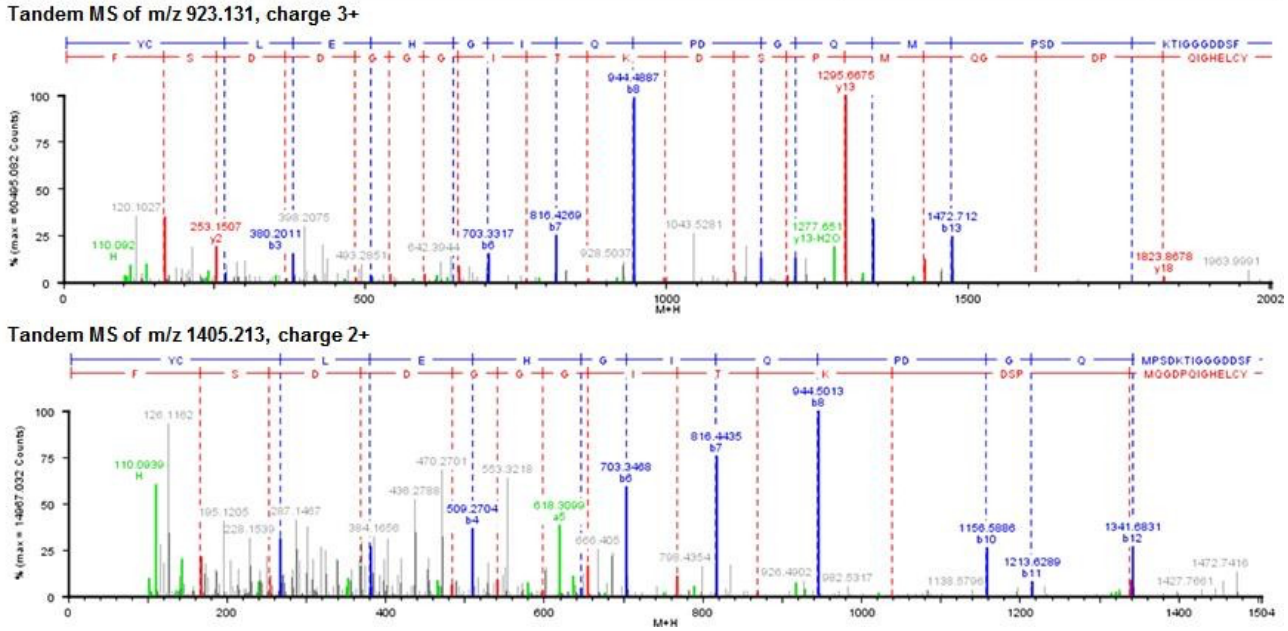

Figure 1. Identification of the peptides containing Lys-40 (Lys-40 peptide, m/z 923.131)and acetylated Lys-40 (surrogate peptide, m/z 1405.213) by ProteinLynx Global Server (PLGS) 2.4.

Table 1. Pepsin digestion efficiency (%, n = 3) for the generation of the Lys-40 peptide (YCLEHGIQPDGQMPSDKTIGGGDDSF).

	1 µg/mL	10 µg/mL	100 µg/mL
DigestEfficiency	45.4 ±1.9	53.2 ±5.2	9 ±2.4

Method Development

Immunoprecipitation was tested with two commercially available antibodies for Lys-40 acetylated α-tubulin: mouse mAb 6-11B-1 and rabbit mAb D20G3. The epitope for 6-11B-1 is located on the α3 isoform of Chlamydomonas axonemal α-tubulin, within four residues of acetylated Lys-40[22,23], but the epitope for D20G3 is unreported. The affinity of these two antibodies with protein A and G magnetic beads were tested, by measuring the recovery of the synthetic surrogate peptide from bovine serum albumin in PBS-T. 6-11B-1 mouse mAb and protein G allowed the highest recovery of the acetylated tubulin peptide and were selected for immunoprecipitation.

Table 2. Comparison of the recovery[a] of 36 pmol acetylated peptide from 100 µL of 1 mg/mL bovine serum albumin in PBS-T.

	ProteinA		ProteinG	
6-11B-1	42%	44%	47%	51%
D20G3	20%	21%	19%	17%

[a] Recovery (%) was calculated according to the following formula: (peak area measured after IP/peak area measured before IP) ×100%.

The immunoprecipitation protocol was adapted for mass spectrometry analysis. The last washing buffer (PBS buffer) was replaced with 50 mM ammonium acetate (pH 7.0) and the elution buffer (100mM glycine-HCl, pH 2.0) was replaced with 100 mM glycine-TFA (pH 2.0), and did not affect the recovery of the immunoprecipitation. Multiple elution up to three volumes wastestedand didn'tincreasetherecovery, either(datanotshown).Thefinaloptimizedprotocol is shown in Figure 2.

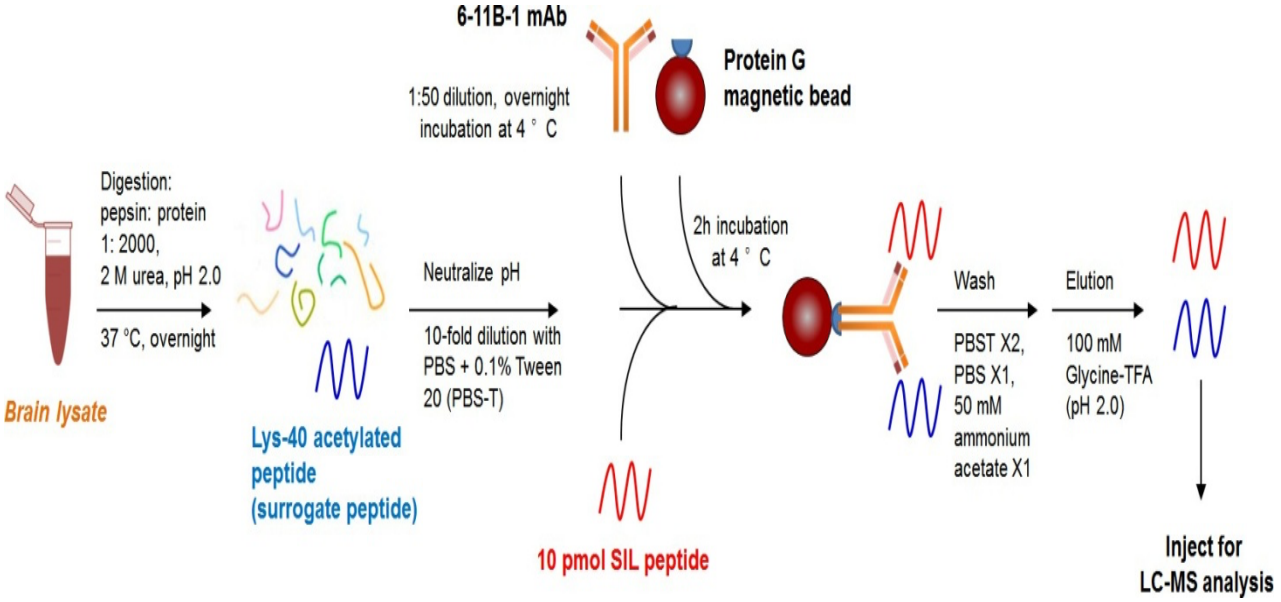

Figure2.The workflow for the immunoprecipitation of Lys-40acetylated peptide (surrogate peptide).

Method Validation

The method was validated with selectivity, linearity, precision and accuracy. Selectivity (n = 3) was validated by analyzing blank samples and spiked samples (1 pmol/mg surrogate peptide and SIL peptide), and chromatograms were compared (Figure 3). No significant interferences from blank matrices were observed, demonstrating that the sample preparation and LC-Msmethodhasthenecessaryselectivitytodifferentiateandquantitateanalytesinthebrain tissue lysate. LLOQ of the method was determined as 1 pmol/mg brain tissue, with a signal-to- noise (S/N) of

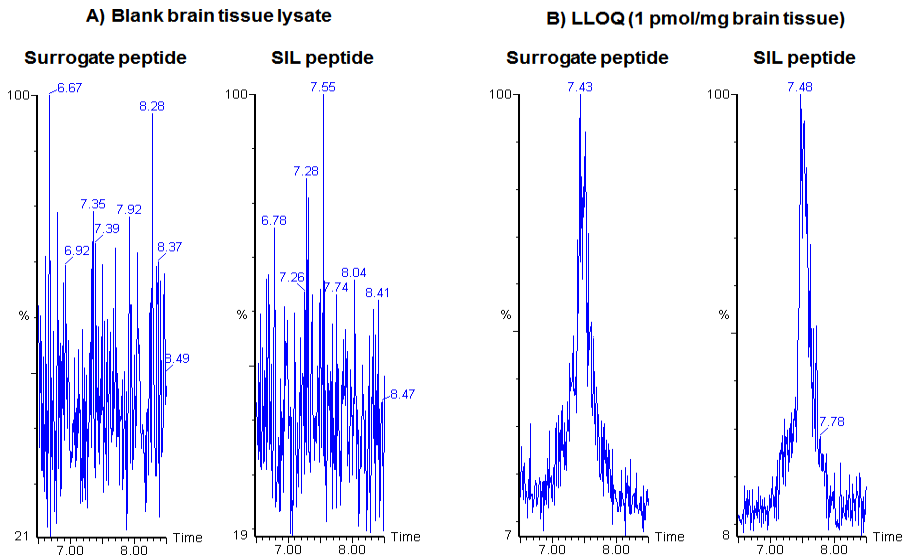

Figure 3. Representative chromatograms of the surrogate and SIL peptide in the blank brain tissue lysate depleted with acetylated tubulin (A), and brain tissue lysate with 1 pmol surrogate and SIL peptide/mg brain tissue (B). Retention times for analytes are shown in minutes.

The linearity was tested over the range of 1.0-100 pmol/mg brain tissue. A calibration curve was made from peak areas of surrogate peptide/SIL peptide, using 1/x weighted linear regressions. The calibration curve, slope, intercept and R^2 value are shown in Figure4. The method allows

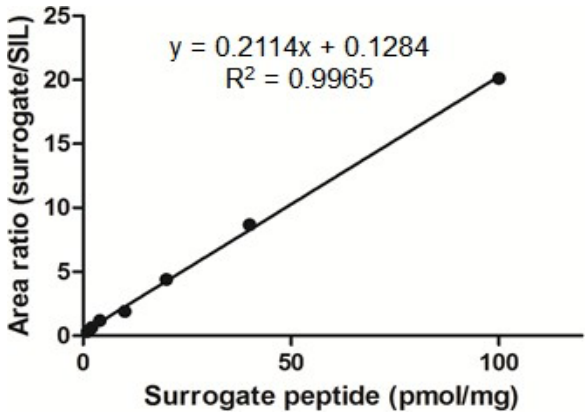

Figure 4. Calibration curve for the surrogate peptide ranging from1 to 100 pmol/mg brain tissue.

for good linearity (R^2> 0.99) within the tested range and the intercept on the y-axis was close to 0, therefore, we use the formula to calculate the concentration of acetylated tubulin in the brain tissue:

Proteinconc (pmol/mg) =SIL amount (pmol)×Response ratio × (1/Digestion efficiency)/ 2 mg Response ratio = Peak area $_{PTMpeptide}$/Peak area $_{SILpeptid}$, SIL amount (pmol) = 10 pmol

The precision and accuracy (n = 3) for acetylated alpha tubulin were tested at 3.0, 30.0 and 75 pmol/mg brain tissue. Precision describes the closeness of a series of measurements from multiple samplings, and was represented by relative standard deviation (RSD); accuracy describes the closeness ofmeasured value to thetruevalue, and was representedbyrelativeerror (RE). Table 3 shows the measurement of each QC sample, and the average, RSD and RE values foreach group.RSDand REareallbelow15%withineach QC groupand meettherequirements from the Guidance for Industry (Bioanalytical Method Validation) by the US FDA.

Table3.Theprecision(RSD)and accuracy (RE)of the analytical method used to quantitate tubulin acetylation from brain tissue lysate.

	QCLow			QC Middle			QCHigh		
	3 pmol/mg			30 pmol/mg			75 pmol/mg		
Measured (pmol/mg)	2.5	3.3	2.4	23.7	33.5	29.2	80.4	72.7	73.2
AVG[a]	2.7 ±0.4			28.8 ±4.0			75.4 ±3.5		
RE[b]	-12.0%			-4.0%			0.5%		
RSD[c]	14.8%			13.9%			4.6%		

AVG: average, RE: relativeerror, RSD: relativest and arddeviation.

Application

Chronic exposure to organophosphate insecticides was observed to lead to impairmentsin cognition and axonal transport, independent of acetylcholinesterase inhibition [24,25,26]. The underlying mechanisms forits neurotoxicity and thetarget proteins, are still unclear. Considering the importance of Lys-40 acetylation in the nervous system, it isimportant to have a specific and selective method to absolutely quantitate this key PTM in brain tissue as a biomarker, toestablish its association with organophosphate exposure conditions including dose, route of administration and duration of time, as well as with cognition defects and alterations in axonal transport.

The validated method was applied to analyze brain tissue collected from rats (n = 3) orally dosed with chlorpyrifos (18mg/kg/every other day) in peanut oil for 30 days with a 45 day washout period. Brain tissue lysate from each rat was prepared and analyzed, and the concentration was calculated with the formula above. The average concentration with in each group was calculated and a student t-test was conducted to compare the difference between control and the chlorpyrifos treatment group. α-tubulin acetylation at Lys-40 was inhibited significantly ($P = 0.0198 < 0.05$) by 55.2 % after exposure to chlorpyrifos (Table 4). Organophosphates (OPs)have been demonstrated to covalently modify a wide variety of proteins, and organophosphorylation has been identified on multiple amino acid residues on tubulin, including Try and Lys [27,28]. The inhibition of tubulin acetylation may result from these covalent modifications, through steric effects for acetylation from inside[9], disruption of tubulin polymerization [29], changes in intermolecular forces or covalent modification in the PTM site.

Table 4. The concentration of Lys-40 acetylated α-tubulin in the rat brain tissue obtained from rats in the control and chlorpyrifos treatment group (n = 3).

	Control	Chlorpyrifostreatment
Lys-40 acetylated α- tubulin	33.5 ±6.6	15.0 ±1.9

Conclusion

Pepsin digestion allowed for the first-time mass spectrometry-based identification of a surrogate peptide for Lys-40 acetylated α-tubulin. An immunoprecipitation-mass spectrometry assay was developed for the first time to quantitate this key PTM in rat brain tissue, and only a small amount of tissue (2 mg) was required. The method was validated with selectivity, specificity, accuracy and linearity within the range of 1-100 pmol/mg brain tissue. An analytical method for this potential cognition relevant biomarker should facilitate studies of neurode generative diseases and neurotoxicity, including the evaluation of pathological progression, the investigation of the mechanisms of actions, and the observation of therapeutic effects.

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