APPLICATION NOTE

Immunoaffinity Mass Spectrometry for Leukotriene Analysis in Brain

Key Information:

- Leukotrienes (LTs) are acute inflammatory mediators that are extremely difficult to isolate and quantify in biological tissues.
- Isolation and quantification of LTs in brain tissue is explored using immunoaffinity capture LC-MS/MS (IA-MS) vs. SPE-MS.
- IA-MS outperformed SPE-MS for LTs allowing for lower level quantitation of these analytes using a simplified, reproducible workflow.

Immunoaffinity Mass Spectrometry for Leukotriene Analysis in Brain

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Introduction

Leukotrienes (LTs) are acute inflammatory mediators and downstream products of arachidonic acid oxidation by the enzyme 5-lipoxygenase (5-LO).[1] Due to their putative role in the pathogenesis of inflammatory diseases such as asthma, neurological diseases, and diabetes, LTs are under investigation to further understand their function in disease progression and to determine their potential value as biomarkers.[2-4] However, their low abundance and rapid clearance make LTs difficult to measure in biological matrices. GC-MS has been successfully used to measure LTs but requires multiple derivatization steps. ELISAs have also been used, but suspected antibody cross reactivity with other isobaric oxylipids raised concern about the accuracy and use of ELISAs.[5]

LTs have been reported to be present at very low levels in normal mouse brain tissue.[6] As a result, extraction methods typically require a concentration step in order to improve analytical sensitivity. Solid phase extraction (SPE) is commonly employed; however, depending on the matrix, this technique can lead to poor recovery and/or poor specificity for LTs that affects sensitivity and reproducibility of the method.

Immunoaffinity (IA) enrichment has multiple benefits over traditional SPE for targeted trace analysis. The specificity of the antibody provides minimal risk of column overloading, which allows for the use of large sample volumes necessary for trace analyte enrichment. Samples are cleaner, which minimizes the problems associated with matrix interferences. The promiscuous nature of antibodies, which is the critical concern being raised about their use in immunoassays, is overcome by the improved specificity and resolving power of the LC-MS/MS instrumentation.[7-8] The combination of these techniques offers an optimal solution for trace biomarker analysis. Accordingly, an IA-MS method was developed to isolate and quantify LTB_4, LTC_4, LTD_4, and LTE_4 in brain tissue homogenates, and this method was compared to an equivalent optimized SPE method.

Methods

Solid Phase Extraction

The optimized SPE was performed using the following procedure:

1. An Oasis Max SPE 96-well plate was conditioned with 500 µl of acetonitrile (MeCN) followed by 25% MeCN in water.
2. Brain homogenate samples (1 ml) were spiked with 25 µl of the Internal Standard solution (50 ng/ml each of LTB_4, LTC_4, LTD_4, and LTE_4 in ethanol), diluted to 2 ml total volume with 0.1 M K_2PO_4 at pH 7.0, and then loaded dropwise onto the SPE plate.
3. The loaded sample plates were washed with 500 µl of 25% MeCN in water followed by 500 µl MeCN neat wash with MeCN and then elution with MeCN/IPA.
4. The analytes were eluted slowly with 500 µl of MeCN/IPA/formic acid (47.5:47.5:5), dried under nitrogen, and reconstituted with 100 µl of 1:1 MeCN/water.

Immunoaffinity Enrichment

In order to measure the cysteinyl LTs (CysLTs) and LTB_4, we prepared two separate IA resins using Cayman's CysLT Monoclonal Antibody and LTB_4 Monoclonal Antibody. These polymer-based IA resins were manufactured in-house using proprietary methods.

Prior to use, the analytical performance of each resin was verified by applying plasma spiked with LTB_4 or LTC_4, running the standard enrichment protocol, and analyzing the eluents by ELISA (Cysteinyl Leukotriene ELISA Kit (Cayman Item No. 500390) and LTB_4 Express ELISA Kit (Cayman Item No. 10009292)). Precision, recovery over linear range, carryover, loading capacity, and specificity of each resin were measured and shown to meet predetermined quality requirements.

Whole brains from healthy C57BL/6 mice were obtained using standard practices and stored at -80°C until use. Brains from three adult (10-week-old) mice and four 2-week-old (p14) mice were analyzed.

IA sample extraction was performed using the following procedure:

1. LTB_4 and CysLT resins were mixed in a 1:1 ratio prior to applying the sample: 250 µl (as slurries in 0.1 M K_2PO_4, pH 7.0) of LTB_4 IA resin and CysLT IA resin were aliquoted into a 2 ml spin column, rinsed with 700 µl of 0.1 M K_2PO_4, pH 7.0, and rinse was removed by centrifugation at 5,000 x g.
2. Brain tissues (flash frozen and stored at -80°C until use) were homogenized in 100 mg/ml in 0.1 M K_2PO_4, pH 7.0, containing 1 mM EDTA and 10 µM indomethacin and centrifuged to produce the supernatants. Homogenization was performed using 2 ml vials containing ceramic beads in the Precellys® Evolution with a preprogrammed sequence (2 x 30 second agitations at 4,500 rpm, 30 second pause between agitations). 1 ml of supernatant was spiked with 25 µl of the Internal Standard solution (50 ng/ml each of LTB_4, LTC_4, LTD_4, and LTE_4 in ethanol), diluted to 2 ml total volume with 0.1 M K_2PO_4, pH 7.0, and homogenate was loaded in 4 x 500 µl portions onto the resin for extraction.
3. The resin was washed with 700 µl of homogenization buffer, and the wash was removed by centrifugation at 5,000 x g. The resin was washed a second time using an additional 700 µl of water followed by centrifugation at 8,000 x g.
4. The target analytes were eluted from the resin with 700 µl methanol (at 5,000 x g) into 10 µl of a proprietary trapping solution that minimizes loss of LTC_4.
5. Samples were dried under nitrogen and reconstituted in 100 µl of 50:50 MeCN/water for analysis.

It should be noted that in order to ensure optimal SPE conditions, several factors must be considered and tested during method development that are greatly minimized with the IA procedure. This provides an important additional understanding of the benefits demonstrated by the IA method over other methods.

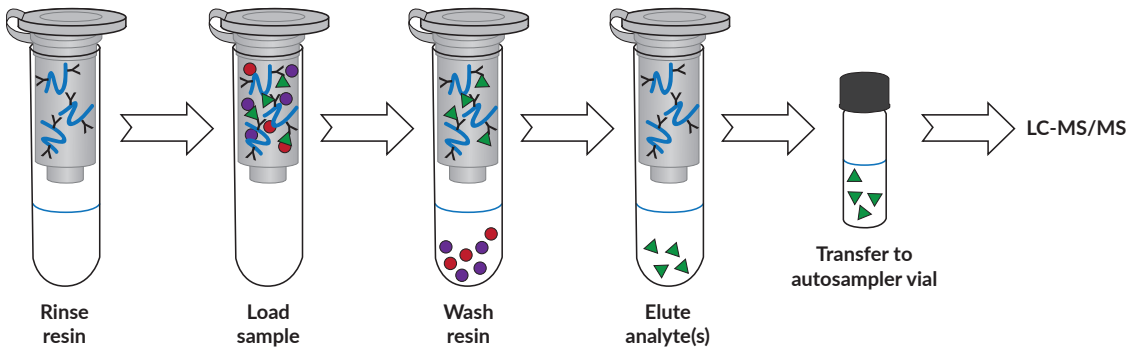

Figure 1. General Immunoaffinity Enrichment Workflow

LC-MS/MS Method

HPLC

Waters ACQUITY UPLC I-Class
Mobile Phase: (A) Water + 0.1% Formic Acid
(B) Acetonitrile + 0.1% Formic Acid
Column: Waters BEH C18 (2.1 x 100 mm, 1.7 µm) @ 30°C
Gradient: 15%B 0 min, 44%B 15 min, 95%B 16 min

MS

Waters Xevo TQ-S micro
Gases/Temps: 600°C Desolvation Temp; 1,000 L/hr Desolvation Gas; 100 L/hr Cone Gas

Parent	Transition	IS	Transition
LTB_4	335.4 > 195.1	LTB_4-d_4	339.4 > 197.1
LTC_4	626.3 > 308.1	LTC_4-d_5	631.3 > 194.2
LTD_4	497.3 > 189.2	LTD_4-d_5	502.2 > 194.2
LTE_4	440.2 > 189.2	LTE_4-d_5	445.1 > 194.2

Table 1. Mass Transition List

Due to the specificity of the IA enrichment method, matrix interferences were not observed and so calibrators were prepared directly in 0.1M K_2PO_4 buffer over the range of 5 pg/ml - 640 pg/ml. Linearity (>0.998), precision (<15%), and recovery (>90%) were within acceptable ranges. The method quantitation limit was determined to be 5 pg/ml for all analytes (plasma) or 0.020 pg/mg of wet tissue weight (brain tissue).

Results

To test the performance of the method in this matrix, whole brains from healthy C57BL/6 mice were obtained using standard practices and stored at -80°C until use. Brains from three adult (10-week-old) mice and four 2-week-old (p14) mice were analyzed in the initial study.

Whole brains were homogenized in a Precellys® Tissue Homogenizer (tissue samples and buffer were added to 7 ml Precellys® Tubes containing 2.8 mm ceramic beads; samples were homogenized for 2 cycles of 30 seconds at 5,000 rpm with a 30 second rest between cycles) and 100 mg of homogenate was used in the extraction procedures. As shown in the summary table below (**Table 2**), the observed levels of LTD_4 and LTE_4 were very similar between extraction methods with mean deviations within 15%. Reported LTC_4 levels were approximately 40% higher when using the IA enrichment method compared to SPE. More significantly, LTB_4 levels were below detection limits when using the SPE extraction procedure but measurable amounts of LTB_4 were observed when IA enrichment was applied. Extraction recovery of LTC_4 was much lower in the SPE extraction method (<50%) as measured by spiked recovery experiments. **Figure 2** shows the results for a representative brain homogenate sample. SPE extraction of the brain sample (top row) resulted in a very low LTC_4 signal and undetectable LTB_4 signal compared to the IA method (bottom row).

	LTE_4 (pg/mg)		LTD_4 (pg/mg)		LTC_4 (pg/mg)		LTB_4 (pg/mg)	
Brain Sample	**SPE**	**IA**	**SPE**	**IA**	**SPE**	**IA**	**SPE**	**IA**
B11	0.059	0.058	0.092	0.103	0.039	0.068	<LOQ	0.032
B12	0.029	0.038	0.083	0.063	0.012	0.026	<LOQ	0.072
B13	0.028	0.034	0.042	0.034	0.024	0.053	<LOQ	0.033
B14	0.027	0.030	0.063	0.045	0.021	0.016	<LOQ	0.023
Average	0.036	0.040	0.070	0.061	0.024	0.041	0.000	0.040
Mean Difference	9.85%		-14.46%		40.66%		100.00%	

Table 2. SPE *versus* IA LC-MS/MS results for brain homogenates

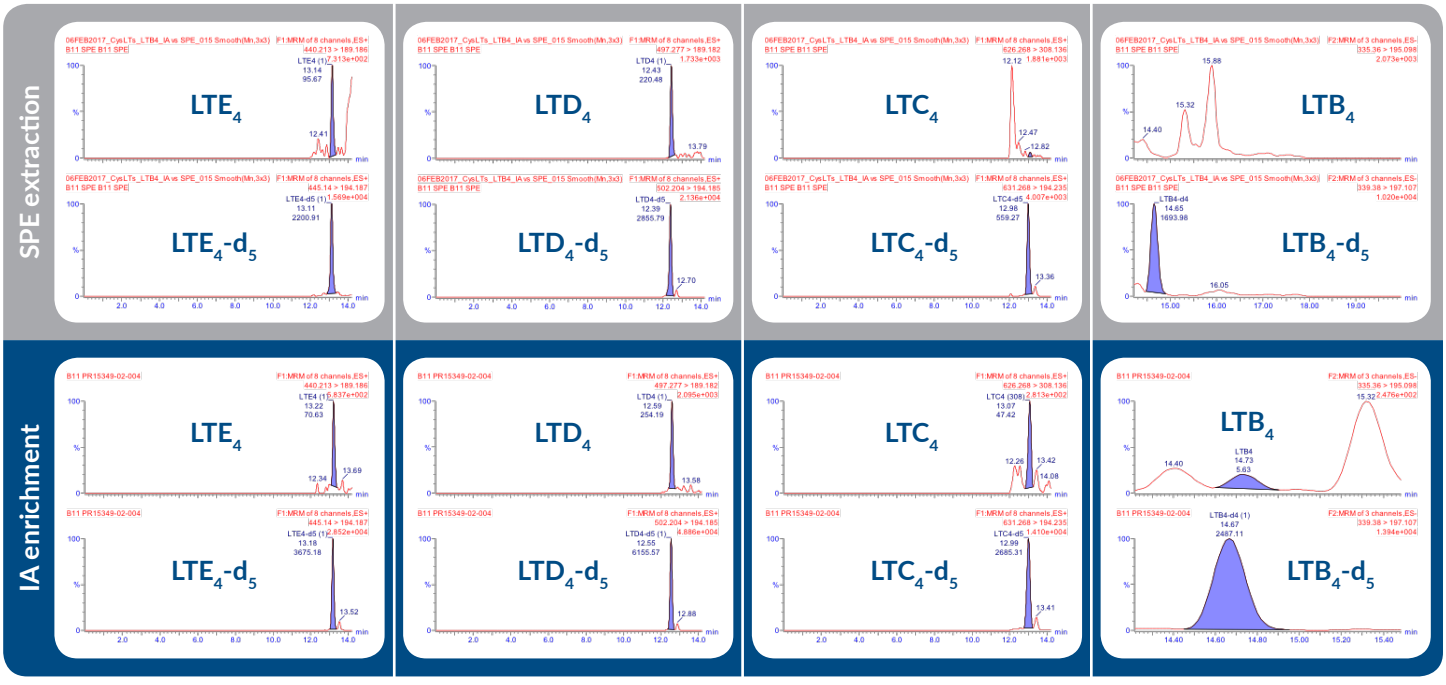

Figure 2. Comparison of SPE extraction (top) *versus* IA enrichment (bottom) for a representative brain sample. Chromatogram pairs are parent and IS MRM signals and analytes from left to right are LTE_4, LTD_4, LTC_4, and LTB_4.

Results *(continued)*

An optimized IA enrichment method was used to compare LT levels in adult (10-week-old) mice to 14-day-old mice. Results are shown in **Table 3** and are reported as pg of analyte per mg wet weight of tissue. Each of the four LTs were detected in all samples. In sample p14_WT-4, the LTC_4 level fell just below the lowest point on the calibration curve, and in this case, the LTC_4 level was estimated by extrapolation from the curve. As shown in **Table 3**, LTB_4 ranged from 0.023–0.188 pg/mg, LTC_4 ranged from <0.020–0.089 pg/mg, LTD_4 ranged from 0.045–0.1085 pg/mg, and LTE_4 ranged from <0.020–0.087 pg/mg. Additionally, LT levels were observed to be lower in the 14-day mice compared to the 10-week mice; however, no conclusions regarding age-related differences in LT levels in mice can be drawn from this preliminary method development study.

Sample ID	LTB_4	LTC_4	LTD_4	LTE_4
p14_WT-1	0.032	0.068	0.103	0.058
p14_WT-2	0.072	0.026	0.063	0.038
p14_WT-3	0.033	0.053	0.034	0.034
p14_WT-4	0.023	BQL	0.045	0.030
AVG	0.040	0.041	0.061	0.040
STD DEV	0.022	0.024	0.030	BQL
adult_WT-1	0.137	0.066	0.109	0.073
adult_WT-2	0.084	0.089	0.069	0.076
adult_WT-3	0.188	0.038	0.107	0.087
AVG	0.136	0.064	0.095	0.079
STD DEV	0.052	0.026	0.023	BQL

Table 3. Summary of Brain LT Data (pg/mg). BQL = Below Quantitation Limit

Conclusions

An IA-MS method was developed for measuring trace LT levels in tissue and compared to SPE. The optimized IA method was comparable to the SPE method for LTE_4 and LTD_4; however, IA enrichment performed better than SPE for the analysis of LTC_4 and LTB_4 in brain homogenate samples. Advantages of the IA enrichment sample prep method over the SPE method include ease of optimization of the workflow, high specificity and high recovery for the desired analytes, and an improved cleanup of the sample from other interfering matrix components that improves sensitivity for low level endogenous analytes. Combined with UPLC-MS/MS, this IA enrichment method allows for the highly sensitive and simultaneous measurement of LTB_4, LTC_4, LTD_4, and LTE_4.

The use of IA-LC-MS/MS analysis of targeted, trace biomarkers provides an opportunity to overcome the challenges of immunoassays and improve methods for research and clinical laboratories. Cayman will continue to develop and introduce IA-MS kits that contain all necessary components (*e.g.*, standards, protocols, and suggested MS methods) for ease of use.

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