Elimination of Matrix Effects Using Mixed-mode SPE Plate for High Throughput Analysis of Free Arachidonic Acid in Plasma by LC-MS/MS

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Introduction

In bioanalysis, matrix interferences present in bio-samples can influence the analysis of interesting compounds. It is well known those interferences may result in matrix effects of ion suppression or enhancement caused by co-eluting interferences on LC-MS/MS. Therefore, it is necessary to consider the elimination of the matrix effect at method development stage.

Arachidonic acid is a ω-6 long chain polyunsaturated fatty acid which is high content in human body originate from dietary animal sources-meat, eggs, dairy, or converted from linoleic acid. Arachidonic acid and its metabolites have a strong biological activity and can regulate a variety of physiological processes, such as the regulation of lipid and glucose, prevention of cardiovascular disease, chemoprevention of cancer cells, improvement of memory.

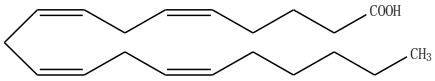

Fig. 1: Chemical structure of arachidonic acid

There are many methods for the detection of arachidonic acid in plasma, such as gas chromatography coupled with mass spectrometry, liquid chromatography with pre-column derivation fluorescence detection, ELISA method. However, these methods are either too complicated to manipulate or with less precision. In contrast, LC-MS/MS has been well accepted as a technique for the determination of arachidonic acid with high sensitivity[1-4]. In order to detect arachidonic acid in plasma by LC–MS/MS, the samples have to be pretreated to remove interferences from the matrices which may result in matrix effect on mass spectrometry[5].

The aim of this study was focused on developing a high throughput and sufficient sample

clean-up method prior to the analysis of arachidonic acid in plasma by LC-MS/MS. Various sample preparation methods including PPT, LLE, single-mode SPE with reversed phase packing material and mixed-mode SPE of Cleanert MAS-M were investigated for removing interferences from plasma.

Experimental

Materials

Arachidonic acid (purity of 99%）, formic acid, ammonia were purchased from Sigma-aldrich (St. Louis, MO, USA). Methanol, acetonitrile and ethyl acetate were HPLC-grade and purchased from Merck (Darmstadt, Germany). Purified water was produced by a Milli-Q Academic System (Millipore, Billerica, MA, USA). Human plasma was obtained from local hospital. Stock solution was prepared by dissolved 10 mg of Arachidonic acid in 100mL of methanol. The stock solution was diluted with a mixture of acetonitrile：water (70：30,v/v) to obtain work solution. Cleanert PPT 96-well plate, Cleanert collection 96-well plate, Cleanert PEP 96-well plate(60mg/well), Cleanert MAS-M 96-well plate(60mg/well) were purchased from Bonna-Agela Technologies (Wilmington, Delaware, USA). Positive pressure SPE manifold, vortex mixer and centrifuge for 96-well plate were purchased from Bonna-Agela Technologies.

Instrumentation

The LC-MS/MS system was consisted of a Shimadzu LC-20A HPLC system with a binary pump and an auto-injector coupled with a triple quadrupole tandem mass spectrometer API4000+ (AB SCIEX, MA, USA). Data acquisition and processing were performed using Analyst software (version 1.5.1) from AB SCIEX. Chromatographic separation was achieved on a Venusil ASB C18 column (3 μm, 150 Å, 2.1 mm×50 mm, Bonna-Agela Technologies) at a column temperature of 30℃ under an isocratic condition with mobile phase of acetonitrile/water (75:25, v/v) at a flow rate of 0.2 mL/min. The injection volume was 5 μL. The target compounds eluted from the HPLC column was introduced directly to the API4000+ and detected by the electrospray ionization interface with negative ion mode for arachidonic acid and phospholipids with positive ion mode, respectively. Quantitative analysis was performed under MRM mode by calculating the peak areas. The optimal MS

parameters were listed in Table 1. An UV detector at 254 nm wavelength was applied for the detection of proteins. The ions were detected by multiple reaction monitoring (MRM), monitoring the [M + H]+ transition of the m/z precursor ion to the m/z of the product ion for arachidonic acid. These MS/MS transitions utilized for analysis were m/z 303/259.1 and 303/205.1.

Table 1: MS parameters

Compounds	t_R/min	Q1	Q3	DP	CE	IS/V	TEM/℃	GS1/Pa	GS2/Pa	CUR/Pa
Arachidonic acid	3.3	303	259.1	-109	-18	-4500	500	55	35	15
		303	205.1	-107	-20					
Phospholipids	4.1	496.3	184.3	63	20	+5500	600	50	50	15

Sample Preparation

5 mL of plasma was spiked with 50 μL of arachidonic acid work solution and vortexed for 30 second to get homogenate samples.

Method A-Protein precipitation

100 μL of plasma sample diluted with 100 μL of 1% formic acid was loaded into each well of Cleanert PPT plate together with 400μL of acetonitrile. The plate was vortexed for 30 second and then set on 96-well SPE plate manifold to elute the target compounds into collection plate, and the eluates were dried at 45℃ under a gentle stream of nitrogen. The residues were reconstituted with 200 μL of acetonitrile: water (70:30,v/v) into each well of collection plate for further analysis.

Method B - Liquid-liquid extraction

100 μL of plasma sample diluted with 100 μL of 1% formic acid was loaded into each well of 96-well collection plate together with 5 μL of methanol. After 30 sec vortex, 500μL of ethyl acetate was added into each well of the plate and then vortexed for 3min. The plate was stood for 1min, and then was centrifuged at 6000 rpm for 5min. The supernatants were transferred into a clean the plate sequentially and were dried at 45℃ under a gentle stream of nitrogen.

The residues were reconstituted by adding 200 µL of acetonitrile:water (70:30,v/v) into each well of the plate for further analysis.

Method C - Solid phase extraction with Cleanert PEP

100 µL of plasma sample diluted with 100 µL of 1% formic acid and loaded into each well of Cleanert PEP plate which was pre-conditioned with 1mL methanol and 1mL water sequentially. The SPE plate was washed with 500 µL of methanol:water (5:95,v/v). The target compounds were eluted with 2mL of 5% ammonia in acetonitrile. The eluates were concentrated at 45℃ under a gentle stream of nitrogen to dryness. The residues were reconstituted with 200 µL of acetonitrile:water (70:30,v/v) for further analysis.

Method D - Solid phase extraction with Cleanert MAS-M

100 µL of plasma sample diluted with 100 µL of 3% ammonium hydroxide was loaded into each well of Cleanert MAS-M plate which was pre-conditioned with 1mL methanol and 1mL water sequentially. The plate was washed with 500 µL of water followed by 500 µL of methanol. The target compounds were eluted with 600 µL of 3% formic acid in acetonitrile. The eluates were concentrated at 45℃ under a gentle stream of nitrogen to dryness. The residues were reconstituted with 200 µL of acetonitrile:water (70:30,v/v) for further analysis.

Results and Discussions

Comparison of sample pretreatment methods for removing phospholipids

It is necessary to remove phospholipids from plasma before injection since it will result in matrix effect on mass spectrometry. Although PPT is a common method in bio-sample preparation, but as shown in Fig.3 a big phospholipids peak appears that may influence the analysis of arachidonic acid. In Method B a traditional LLE technique was used for removing phospholipids from plasma. However, it is clear from Fig.3 this method is not able to eliminate phospholipids effectively. The results of Method C indicates that single-mode SPE with non-polar interaction is suitable for effectively remove of phospholipids from plasma. as shown in Fig.3 that phospholipids may co-elute with target compound. The results obtained from Method D demonstrates with Cleanert MAS-M, a mixed-mode SPE plate offering reversed phase, anion exchange and cation exchange interactions, the influence of phospholipids on MS is eliminated significantly. Because of the amphoteric property,

phospholipids are adsorbed strongly on to the plate while the target compounds are passed through the plate.

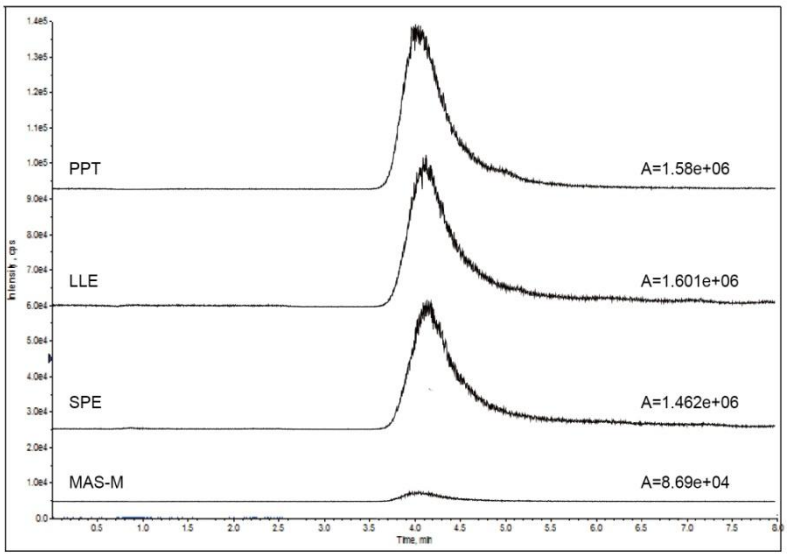

Fig. 3: Chromatograms and peak area of phospholipids in plasma treated with four clean-up methods. Where PPT is Method A, LLE is Method B, SPE is Method C and MAS-M is Method D.

Comparison of sample pretreatment methods for removing proteins

Proteins with larger molecular weight have similar retention behaviors as arachidonic acid on reversed phase HPLC column. It would cause matrix effect on the determination of arachidonic acid by LC-MS/MS. As shown in fig. 4, there are two big peaks appeared in Method A while the chromatograms of the rest of sample pretreatment methods are effective enough to remove proteins from plasma. Combining with the capability for removing phospholipids, Method D with Cleanert MAS-M plate was selected for high throughput sample clean-up of arachidonic acid before analysis by LC-MS/MS.

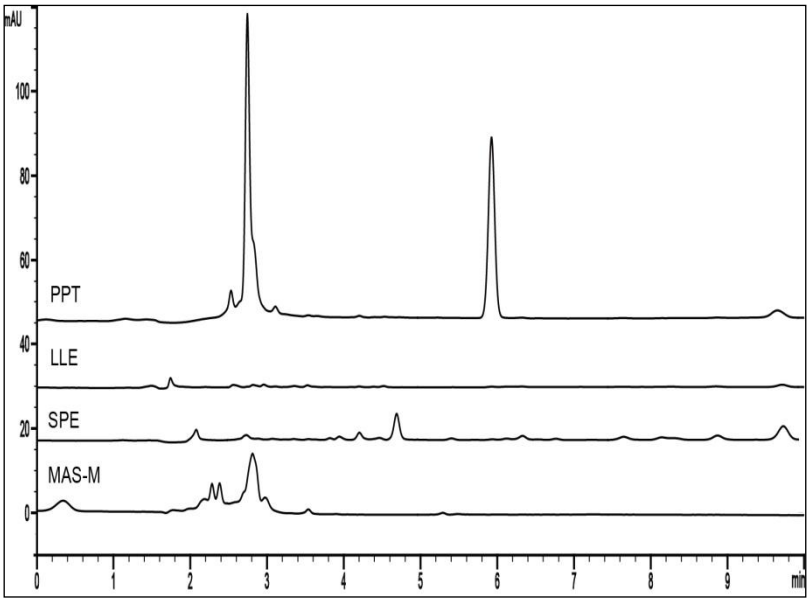

Fig. 4: Chromatograms of proteins in samples treated with four clean-up methods. Where PPT is Method A, LLE is Method B, SPE is Method C and MAS-M is Method D.

Cleanert MAS-M is packed with the combination of two different resins: reversed phase and anion exchange interactions are applied to extract arachidonic acid from plasma, while cation exchange utilized to avoid co-elution of phospholipids and proteins.

3% ammonium hydroxide used to dilute plasma is critical to improve the recovery of arachidonic acid, because it will released the target compounds from proteins by ionizing arachidonic acid. The ionized arachidonic acid can be adsorbed strongly on Cleanert MAS-M during sample loading process. Since arachidonic acid is retained on the plate by anion exchange interaction, methanol can be used to wash out more hydrophobic impurities without the loss of arachidonic acid. 600 µL of 3% formic acid in acetonitrile was used to elute arachidonic acid while the phospholipids and proteins were remained on the plate.

The chromatographic behaviors and recoveries of arachidonic acid with various sample pretreatment methods

As shown in fig. 5, there are significant peak shifts of arachidonic acid when the plasma samples were pre-treated with Method A (peak a), Method B (peak b) and Method C (peak c). The reason of this phenomenon may due to the active groups of the HPLC column were blocked by the co-eluted phospholipids and proteins of plasma resulted in the less retention of target compound on the column. Since phospholipids and proteins are removed from plasma

effectively with Method D there is no peak shift on arachidonic acid. Another interesting phenomenon is that the concentrations of arachidonic acid in blank plasma samples pre-treated with Method A (peak a), Method B (peak b) and Method C (peak c) are very high compare with that of Method D (peak e) that may cause by the ion enhancement during analysis of LC-MS/MS.

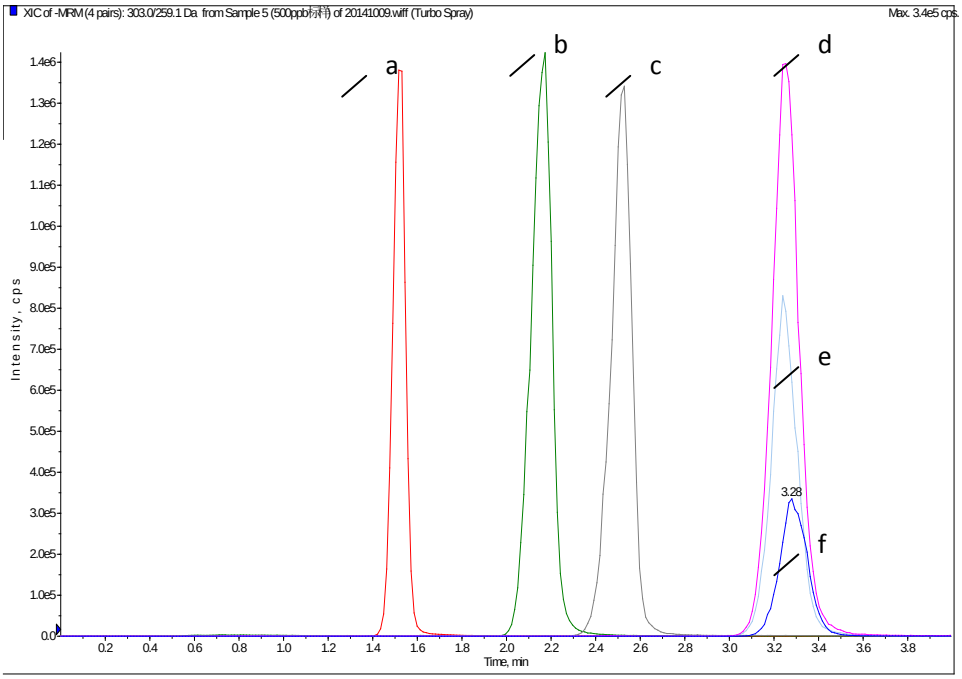

Fig. 5: Chromatograms of arachidonic acid in different samples. a- unspiked plasma of Method C. b- unspiked plasma of Method B. c- unspiked plasma of Method A. d- spiked plasma, 500ng/mL, of Method D, e- unspiked plasma of Method D. f- arachidonic acid standard solution at 500 ng/mL.

The recoveries of arachidonic acid from various sample pretreatment methods with two concentration levels at 500ng/mL and 2μg/mL are listed in Table 2. As demonstrated in Fig. 3 the elimination of phospholipids and proteins is not satisfactory by Method A, Method B and Method C comparing with Method D, the matrix effect caused by phospholipids and proteins is the main factor resulting in various recoveries of arachidonic acid. The recoveries and precision of the Method D with Cleanert MAS-M plate are summarized in table 2. The average recoveries are in the ranges of 99.38%~103.21% with RSD ranged from 5.17% to 5.34%.

Table 2: Recoveries of arachidonic acid in spiked plasma n=5

Concentration	PPT		LLE		Single-mode SPE		Mixed-mode SPE	
	Recoveries (%)	RSD (%)	Recoveries (%)	RSD (%)	Recoveries (%)	RSD (%)	Recoveries (%)	RSD (%)
500ng/mL	129.32	11.14	85.48	55.90	132.95	19.60	103.21	5.17
2μg/mL	130.42	2.06	67.05	82.21	94.66	9.21	99.38	5.34

Applications of the Proposed Methods on human plasma

The optimized clean-up method using Cleanert MAS-M plate coupled with LC-MS/MS was applied to analyze free arachidonic acid in human plasma samples obtained from local hospital. The results are listed in table 3.

Table 3: Free arachidonic acid in some human plasma samples

Sample No.	1	2	3	4	5	6	7	8
Concentration (μg/mL)	1.51	0.83	1.15	0.98	1.83	2.11	2.18	1.82

This method was applied in medical research center of local hospital to determine more than 100 plasma samples from patients with coronary heart disease and healthy people. With the high throughput sample clean-up method utilizing 96-well plate, the testing efficiency was improved and well received by the researchers from local hospital. The results showed the average concentration of free arachidonic acid in the plasma from patients was lower than that from healthy people, which fit the law of pathology.

Conclusions

A mixed-mode SPE plate of Cleanert MAS-M can be used for eliminating matrix effect of phospholipids and proteins prior to the analysis of arachidonic acid in plasma by LC-MS/MS. A sufficient recovery with acceptable precision are obtained. The method is successfully

applied for the assay of arachidonic acid in patient's plasma samples.

This study also gives a clue that the sample clean-up method with Cleanert MAS-M plate could be adapted to sample pretreatment of hydrophobic analytes in plasma which are usually co-eluted with phospholipids and proteins on reversed phase HPLC column.

References

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