The Potential of SPE for Fractionation of Serum Metabolome into Phospholipids and Other Metabolites

Introduction

The high concentration of phospholipids (PLs) in blood serum is a frequent limitation in metabolomic analysis based on mass spectrometric detection, mainly owing to their ionization suppression effects. Thus, PLs mask the detection of less-abundant metabolites that may be potential biomarkers in clinical applications. This justifies a fractionation step for their removal when dealing with analysis of less-abundant metabolites. On the other hand, metabolism of PLs has been closely associated with different pathologies, such as obesity, cancer disorders, atherosclerosis, diabetic nephropathy, kidney failure, arterial hypertension, neurodegenerative and neurological disorders such as Alzheimer’s and Niemann-Pick’s diseases, metachromatic leukodystrophy, as well as multiple sclerosis, among others. For this reason, the determination of PLs is of enormous clinical interest.

PL Enrichment vs. Interference Removal

Two important points can be derived from the above discussion: First, when PLs are the target analytes, it is necessary to isolate them from other sample components, such as proteins, sugars, and other small molecules, that interfere with the chromatography of the PLs. Second, removal of PLs is necessary with MS detection when they are not the target analytes.

Among the techniques that are used in the clinical field for analyte enrichment or interference removal, solid-phase extraction (SPE) is arguably the most popular. This is due to the high efficiency, easy handling, affordable price, and wide variety of commercially available sorbents with very different selectivities and particle compositions from which the analyst can choose. Versatility is also an important characteristic. One such sorbent that meets these criteria is HybridSPE®-Phospholipid.

The purpose of this study was to investigate the potential of HybridSPE-Phospholipid for the selective isolation and characterization of two serum fractions: one that contains all of the PLs in the sample (an enrichment application), and one where the PLs are removed to enable determination of other, non-PL, metabolites (an interference removal application).

Experimental

An Agilent 1200 Series LC system on-line coupled to an Agilent 6540 UHD QqTOF hybrid accurate mass spectrometer equipped with an electrospray ionization (ESI) source was the platform for this analysis. MassHunter Workstation software (Agilent) was used to process raw MS data, including feature extraction, molecular formula generation, and database searching.

HybridSPE-Phospholipid Method

Both cartridge and plate formats of the HybridSPE-Phospholipid possess a top frit that acts as a filter for the physical retention of precipitated proteins while allowing metabolites to pass through. The top frit also impedes flow of the sample before vacuum application. The bed comprises zirconia-coated silica particles that selectively retain the PLs. The cartridge format was used in this study with the following protocol:

1. Load 300 µL of serum and 700 µL of 1% (v/v) formic acid in acetonitrile to the top of the HybridSPE-Phospholipid cartridge (Supelco Cat. No. 55269-U). The acetonitrile acidified with 1% formic acid added to the serum not only acts as a precipitation agent, but also as a modifier to inhibit the interaction of most acidic compounds with Zr sites on the particles.
2. Vortex cartridge 30 seconds, rest cartridge for 2 minutes.
3. Apply vacuum, collect fraction FA1 (non-retained, flow-through).
4. Add 1 mL of 1% (v/v) formic acid in acetonitrile to the cartridge, apply vacuum, collect fraction FA2.
5. Add 1 mL pure acetonitrile to the cartridge, apply vacuum, collect fraction FA3.
6. Add 1 mL of PL elution solvent (methanol, ethanol, or acetonitrile) containing 5% (v/v) ammonium hydroxide to the cartridge, apply vacuum, collect PL fraction.

LC-QqTOF MS/MS Analysis of Serum Fractions

All chromatographic parameters are described in Figure 1. A 20 µL aliquot of the prepared sample was injected into the LC and separated on a reversed-phase (C18) column. Two different chromatographic gradient profiles were programmed for independent analysis of the individual (FA1, FA2, and FA3), combined (FA), and PL fractions. Accurate mass spectra were acquired in the m/z range 100-1100, typical for metabolomics experiments, at 3.5 spectra/sec. Analyses were carried out both in positive and negative ionization modes with the appropriate continuous internal calibration. Collision energy was varied from 15 to 30 eV to obtain different MS/MS profiles which should support PL identification.
Results and Discussion

The strategy selected for this fractionation approach was based on the selective retention capability of PLs on the zirconia-coated silica particles that comprise HybridSPE-Phospholipid, and absence of retention by those particles of the wide range of basic, neutral, and acidic compounds (e.g., metabolites) in serum. The packed-bed filter/frit assembly acts as an effective filter to facilitate the simultaneous removal of both PLs and precipitated proteins during the extraction process.

The flow-through portion, FA1, together with the two rinsing solutions, FA2 and FA3, showed total absence of PLs indicating the HybridSPE-Phospholipid sorbent was very effective at removing PL interferences. Additionally, both negative and positive ionization modes showed fractions FA2 and FA3 to be essentially devoid of molecular entities of any type. This demonstrated an important feature of the HybridSPE-Phospholipid sorbent: It does not deplete the sample of compounds other than phospholipids.

A caveat, however, the acetonitrile crash solvent must be acidified with 1% (v/v) formic acid to inhibit the interaction of most acidic compounds with Zr sites on the particles.

Elution of PLs from the cartridge was assayed by methanol, ethanol, or acetonitrile [in all cases the eluent contained 5% (v/v) ammonium hydroxide as modifier]. Figure 2A shows the Venn diagram of the molecular entities detected in the eluates using the negative ionization mode, in which the better performance of methanol as eluent is clear.

The effective fractionation of PLs and non-PLs metabolites in serum is shown by the Venn diagram in Figure 2B. The two fractions had no molecular entities in common. This behavior is corroborated in Figure 1, which corresponds to the ion chromatograms extracted from LC-TOF/MS monitoring of lysophosphatidylcholine (LPC) 18.0 (m/z 568.3619) and arachidonic acid (m/z 320.2351) in the FA and methanol-eluted fractions in negative ionization mode. The target PL was not detected in the FA, thus supporting the highly effective retention of PLs by the HybridSPE-Phospholipid cartridge. On the other hand, other metabolites elute unretained with the flow-through sample portion and rinsing solutions, free from PLs.

Conclusion

From these results, it can be concluded that this SPE protocol using HybridSPE®-Phospholipid followed by LC-MS/MS is useful for global metabolome analysis by fractionation, here into non-PL and PL metabolites. Therefore, the multipurpose use of HybridSPE-Phospholipid cartridges commonly used for interference removal has been demonstrated.

Complete details on this research and the PLs detected and confirmed by MS/MS, can be found in the reference.
Figure 2. Venn Diagrams with the Count of Molecular Entities Detected

- Methanol: 107 entities
- Ethanol: 24 entities
- Acetonitrile: 17 entities

107 entities detected in all three solvents.

22 entities detected only in methanol.

2 entities detected only in ethanol.

2 entities detected only in acetonitrile.

15 entities detected in methanol and ethanol.

2 entities detected in methanol and acetonitrile.

0 entities detected in ethanol and acetonitrile.

Reference

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