Targeted Analysis of Phosphorylated Metabolites in Biological Samples by HILIC–LC–MS

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Nucleotides, deoxynucleotides, and coenzymes comprise a wide range of phosphorylated metabolites with zwitterionic nature and high polarity. They constitute a family of compounds that participate in key metabolic pathways, such as glycolysis, pentose phosphate pathway, tricarboxylic acid cycle, and synthesis of nucleic acids. Due to their physicochemical properties, separation and sensitive quantification by LC–MS/MS without using ion-pairing reagents is challenging.

Historically, hydrophilic interaction liquid chromatography (HILIC) has demanded high concentrations of buffers in mobile phase to achieve better retention and separation efficiency for the analysis of phosphorylated metabolites, especially for di- or triphosphates (1). However, a high concentration of salts in the mobile phase suppresses transfer of metabolites to the gaseous phase in electrospray ionization-mass spectrometry (ESI-MS), which results in poor sensitivity and contamination in the ion source. Previous studies have shown that medronic acid binds to the active sites of trace metal in stainless steel tubing of LC, which prevents undesired interactions of phosphorylated compounds with the active sites (2). As a result, the addition of medronic acid in the mobile phase significantly improves peak shapes (less tailing), thus boosting the separation efficiency.

In this application, we describe the methodology applied to targeted analysis of various biological samples such as human *in vitro* differentiated adipocytes (3), plant tissue (*Arabidopsis thaliana* leaves) (4), or mouse skeletal muscle using polymer-based iHILIC-(P) Classic HILIC columns.

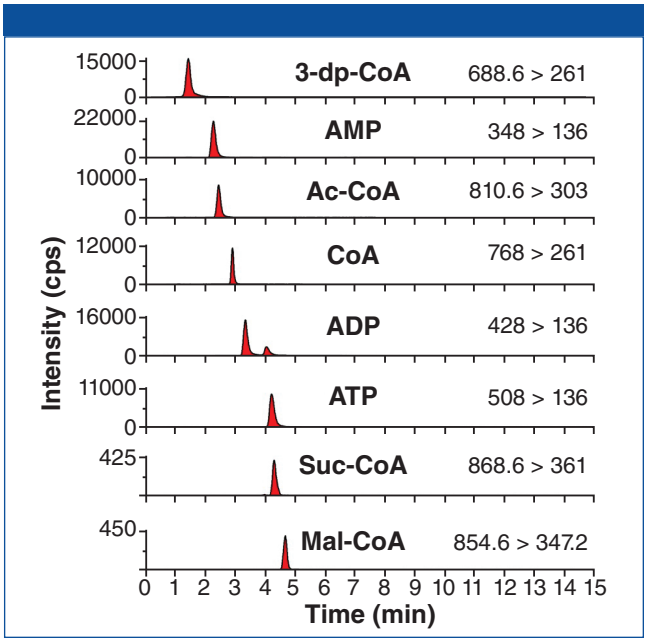

Figure 1: MRM chromatograms from 1 µM mixture of standards in 50% methanol. Column: 50 × 2.1 mm iHILIC-(P) Classic. Elution: Gradient 2.

Experimental

Sample Preparation:

1) Human *in vitro* differentiated adipocytes were lysed at day 14 post-induction of differentiation in 1 mL of 90:10 (v/v) methanol–H_2O solution containing 0.5 µmol/L creatine-D_3 as an internal standard, shaken with metal beads at 30 kHz for 3 min, and centrifuged at 14,000g for 10 min. The supernatant was evaporated and reconstituted in 50 µL of 50:50 (v/v) methanol–H_2O. Targeted metabolites: phosphocreatine, creatine.

2) Rosette leaves (10 mg) of *Arabidopsis thaliana* were extracted with 250 µL of ice-cold extraction medium (chloroform–methanol, 3:7) and incubated at -20 °C for 2 h. Thereafter, 10 µL 50 µM UDP-Glc-$^{13}C_6$ was added to each sample as an internal standard. Samples were then extracted twice with 200 µL of ice-cold water and the aqueous layers combined and dried in a freeze-dryer. The dried samples were dissolved in 50 µL of 50:50 (v/v) methanol–H_2O and diluted 10-fold with the same solvent before the analysis by LC–MS/MS. Targeted metabolites: UDP-Glc.

3) Mouse skeletal muscle (20 mg) was extracted with 500 µL of 90:10 (v/v) methanol–H_2O solution containing 1 µM labelled standards (creatine-D_3, ADP-$^{15}N_5$, ATP-D_4) as in Method 1. Targeted metabolites: 3-dp-CoA, AMP, ADP, ATP, Ac-CoA, CoA, Suc-CoA, Mal-CoA, cyclocreatine, creatine, β-GPA, phosphocreatine.

LC–MS/MS System: An Agilent 1290 UHPLC system with an Agilent 6490 triple quadrupole. Analytes were ionized in an electrospray source operated in both positive and negative mode. The source and gas parameters were set as follows: ion spray voltage at -3.5 kV (+4.0 kV in positive), gas temperature at 150°C, drying gas flow at 11 L/min, nebulizer pressure at 20 psi, sheath gas temperature at 350 °C, sheath gas flow at 12 L/min, and fragmentor at 380 V.

HILIC Separation:

Columns:

1) 150 × 2.1 mm, 5-µm, iHILIC®-(P) Classic (P/N 160.152.0520, HILICON); Flow rate: 0.2 mL/min

2) 50 × 2.1 mm, 5-µm, iHILIC-(P) Classic (P/N 160.052.0520, HILICON); Flow rate: 0.35 mL/min

Eluents:

A) 10 mM ammonium acetate in water (pH 6.8) with 5 µM medronic acid

B) 10 mM ammonium acetate (pH 6.8) in 90:10 (v/v) acetonitrile–water

Column Temperature:

40 °C

Table 1: Gradient programs for separation of phosphorylated metabolites with iHILIC-(P) Classic

15-cm Column		5-cm Column			
Gradient 1		Gradient 2		Gradient 3	
Time (min)	% B	Time (min)	% B	Time (min)	% B
0	90	0	85	0	95
15	30	5	60	3	90
18	30	7	30	8	30
19	90	8	30	9	30
27	90	9	85	10	95
		15	85	15	95

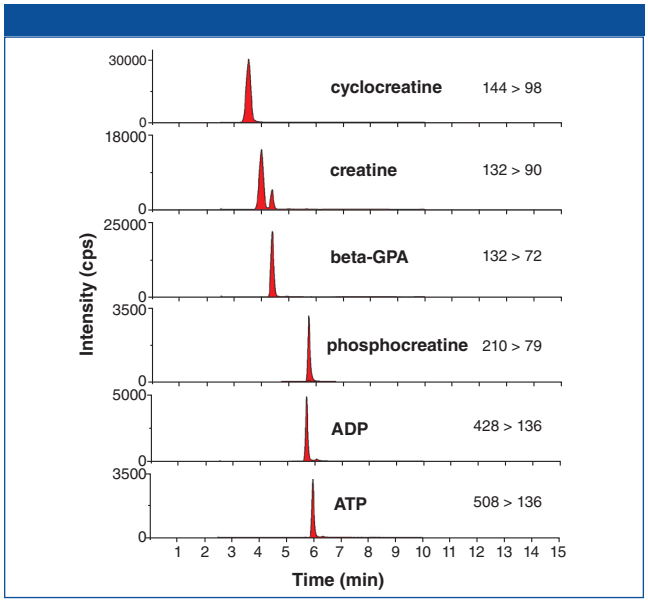

Figure 2: MRM chromatograms from 1 µM mixture of standards dissolved in 50% methanol. Column: 50 × 2.1 mm iHILIC-(P) Classic. Gradient program 3. Phosphocreatine detected in negative ESI.

Results and Discussion

The developed gradient elution methods with iHILIC-(P) Classic columns were optimized to fit the needs of the specific sample matrix and metabolites of interests. Initially, we have been using a 150-mm column and Gradient 1 program (Table 1) for quantification of creatine and phosphocreatine (3) and UDP-Glc (4). However, we discovered that a 50-mm iHILIC-(P) Classic column allows the separation of many phosphorylated metabolites within a 15-min run (Figure 1) whilst retaining a similar separation efficiency as the 15-cm columns. These findings significantly helped us to increase the analysis throughput.

Moreover, by fine-tuning the gradient profile, we managed to separate creatine analogues and their isomeric compounds-β-guanidinopropionic acid (β-GPA) and creatine that share the MRM transition of 132>90. Thus, baseline separation is needed, as shown in Figures 2 and 3.

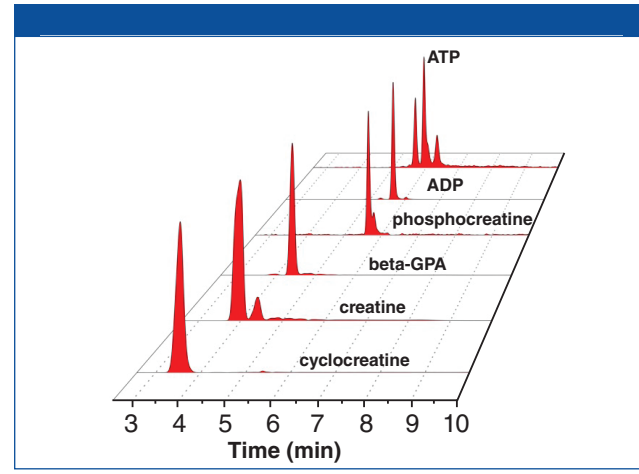

Figure 3: MRM chromatograms from a skeletal muscle extract (10× diluted with 50% methanol). Column: 50 × 2.1 mm iHILIC-(P) Classic. Elution: Gradient 3.

Conclusion

The HILIC–MS methods described in this application are generic for the analysis of polar metabolites in targeted and nontargeted metabolomics. It is straightforward and fast in both sample preparation and separation, which empowers high-throughput analysis.

References

(1) Logotheti, M.; Theochari, K.; Kostakis, M.; Pasias, I. N.; Thomaidis, N. S. Development and Validation of a HILIC-UV Method for the Determination of Nucleotides in Fish Samples. *Food Chemistry* **2018**, *248*, 70–77. DOI: 10.1016/j.foodchem.2017.12.040

(2) Hsiao, J. J.; Potter, O. G.; Chu, T-. W.; Yin, H. Improved LC/MS Methods for the Analysis of Metal-Sensitive Analytes Using Medronic Acid as a Mobile Phase Additive. *Anal. Chem.* **2018**, *90*, 9457–9464. DOI: 10.1021/acs.analchem.8b02100

(3) Maqdasy, S.; Lecoutre, S.; Renzi, G.; et al. Impaired Phosphocreatine Metabolism in White Adipocytes Promotes Inflammation. *Nat. Metab.* **2022**, *4*, 190–202. DOI: 10.1038/s42255-022-00525-9

(4) Wang, W.; Viljamaa, S.; Hodek, O.; Moritz, T.; Niittyla, T. Sucrose Synthase Activity is Not Required for Cellulose Biosynthesis in Arabidopsis. *Plant J.* **2022**, *110*, 1493–1497. DOI: 10.1111/tpj.15752

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