Ultra-Sensitive LC-MRM Based Method for Rapid and High-Throughput Screening of Sugar Phosphate Isomers in Cell Culture

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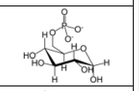
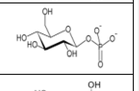
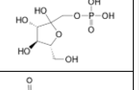
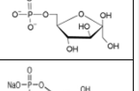
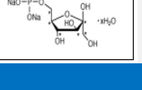
Overview

Introducing robust, ultra-sensitive and accurate LC-MS/MS method for separating Sugar phosphates isomers to perform quantitative high throughput screening.

Introduction

Sugar phosphates isomers are important intermediates of central carbon metabolism with roles in glycolysis, pentose-phosphate pathway, tricarboxylic acid cycle and other biosynthesis pathways. They are used to store or transfer energy and form the backbone of DNA and RNA as well as being involved in various metabolic inherited diseases (1). However, the effective analysis of these compounds in biological samples by liquid chromatography/ mass spectrometry (LC/ MS) is often problematic because of their poor chromatographic retention and separation properties in reversed-phase LC, coelution, and the ionization suppression in ESI. In this study we applied high throughput screening method for the analysis of sugar phosphates in biological samples using ultra-sensitive LC-MS.

Sugar Phosphates	Molecular Formula	Molecular Weight	XLogP3	Precursor m/z	Product m/z	Structure
Glucose 6-Phosphate (G_6P)	$C_6H_{13}O_9P$	260.14	-4.2	259.0224	96.9 78.9	
Glucose 1-Phosphate (G_1P)	$C_6H_{13}O_9P$	260.14	-3.8	259.0224	96.9 78.9	
Fructose 6-Phosphate (F_6P)	$C_6H_{13}O_9P$	260.14	-4.3	259.0224	96.9 78.9	
Fructose 1-Phosphate (F_1P)	$C_6H_{13}O_9P$	260.14	-4.3	259.0224	96.9 78.9	
Fructose 6-Phosphate-13C (F_6P13C)	$(_{13}C)_6H_{11}O_9P\bullet_2Na$	310.06	-4.3	264.61	96.9 78.9	

Methods

Method was developed using Hydrophilic interaction chromatography (HILIC) technology coupled to **SCIEX Triple Quad 7500 MS/MS system** for separation of sugar phosphate isomers. A set of four sugar phosphate isomers: glucose-1-phosphate, galucose-6-phosphate, fructose-1-phosphate, Fructose-6-phosphate were separated and analyzed by LC-MS. Spectra were collected using MRM scan mode then evaluated for diagnostic fragments. Protein precipitation Extraction technique was applied for extraction of metabolites using high throughput screening (HTS) to assess pharmacological and biological activity of chemical hits at different concentrations.

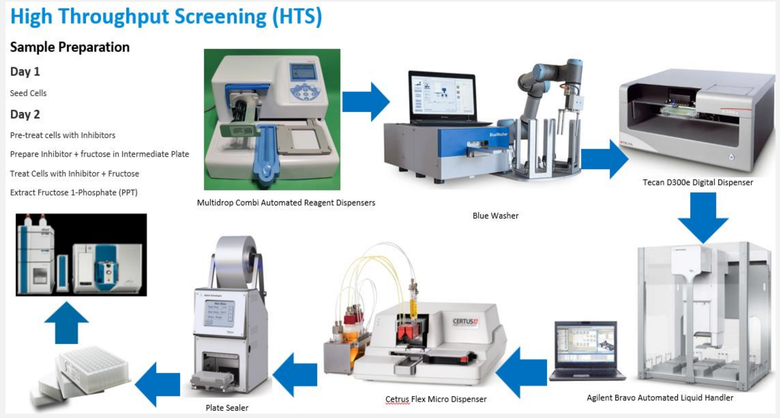
LC Method

Shodex, Polymer-based Hydrophilic Interaction Chromatography (HILIC) VT-50 2D (150 mm) VT-50G 2A (10 mm) Guard column; Injection Volume: 5 µL; Column Temperature: 60 ℃
Mobile Phase A: 20 mM Triethyl Ammonium Acetate, pH= 5.2; Mobile Phase B: Acetonitrile
Isocratic Elution: 94% Mobile Phase A; 6% Mobile Phase B for 15 Minutes; Flow rate: 0.3 mL/min

MS/MS Method

Source Parameters	TEM (400˚C)	IS (+) (2500 V)	CAD (8 psi)	CUR (40 psi)	Gas1 & Gas2 (60 psi)
Compound Parameters	**EP (V)**	**CE (V)**	**CXP (V)**	**Dwell Times (ms)**	
F1P 259.0/96.9	-10	-20	-10	100	
F1P 259.0/78.9	-10	-50	-10	100	
G1P 259.0/96.9	-10	-20	-10	100	
G1P 259.0/78.9	-10	-50	-10	100	
F6P 259.0/96.9	-10	-20	-10	100	
F6P 259.0/78.9	-10	-50	-10	100	
G6P 259.0/96.9	-10	-20	-10	100	
G6P 259.0/78.9	-10	-20	-10	100	
F6P13C 264.6/96.9	-10	-20	-10	100	
F6P13C 264.6/78.9	-10	-50	-10	100	

Sample Preparation Method

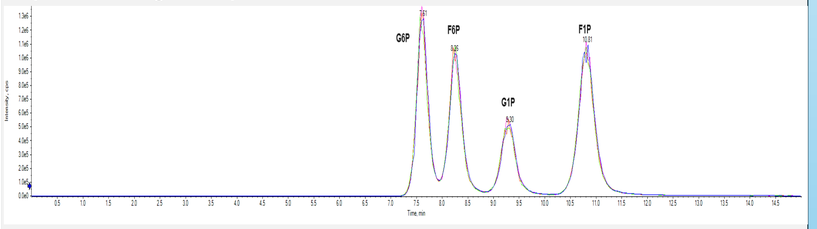


Results

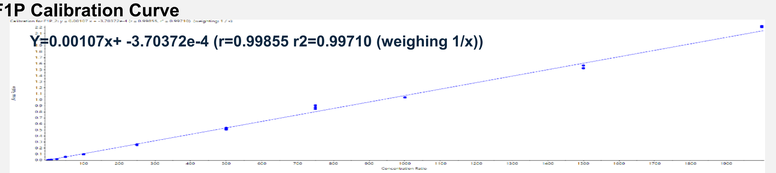
Different chromatographic columns and mobile phases were used to develop a robust LC/MS-based method for the selective and sensitive analysis of sugar phosphates from cell culture with no interferences from their isomers and any need for using ion pairing reagents and derivatization. It was found that the HILIC column provided an improved selectivity for efficient baseline separation of sugar phosphates isomers. Analysis metrics linearity of this newly developed method provided appropriate response and linearity with correlation coefficients (r) greater than 0.98 over concentration range of 1 to 2000 ng/ml. The selectivity and sensitivity of method was evaluated by analyzing blank cell medium and LLOQ sample (1 ng/mL). No peak was observed at or around the retention time for each metabolite following injection of ULOQ (2000 ng/mL), which indicated a carryover-free assay

The precision and accuracy were observed at CV < 15% for all compounds across all calibration levels, samples and QC material used and good stability was exhibited for up to 3 days. The screening involved approximately 120 drug candidates in triplicate, with reference compounds included in each of the forty 96-well plates. The average signal window was 8.9-fold, the average Z prime was 0.77, and the Minimum Significant Ratio (MSR) was 1.9. These HTS statistical data demonstrate the robustness and reproducibility of the assay. The developed LC-MS/MS methods with the goal of being sensitive, specific, and accurate addressed the various issues that can occur for the analysis of sugar phosphates with the choice of column, mobile phase and sample preparation used being of considerable importance. The method developed are optimized and being determined for ease of use for high throughput analysis without any chromatographic issues as these compounds a can be very challenging.

Separation of Sugar Phosphate Isomers

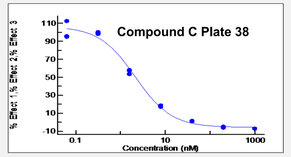


F1P Calibration Curve



Testing inhibition effect of compounds against human KHK in HepG2 Cells

Compound	IC50 (nM)
Compound R Plate 38	94.4
Compound A Plate 38	20.4
Compound B Plate 38	41.5
Compound C Plate 38	2.1



Conclusions and Summary

We developed and applied high throughput screening sample preparation coupled to LC-MRM analytical method allowing the separation and detection of structural isomers to evaluate inhibition effect of compounds against human KHK in HepG2 cells.

References

(1)Anna Stincone et al., Biol Rev Camb Philos Soc, 90 (3), 927-963 (2015)

COI: The authors declare no competing financial interest.