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Determination of energy metabolites in cancer cells by porous graphitic carbon liquid chromatography electrospray ionization mass spectrometry for the assessment of energy metabolism

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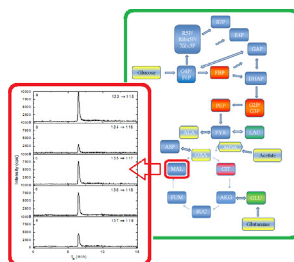
HIGHLIGHTS

- All types of sugar metabolites can be investigated in one run on graphitic stationary phase.
- Method development for acidic metabolites of energy metabolism using a single LC–MS run.
- Study of 15 acidic energy metabolites on a PGC column using common eluents.
- Lactate, acidic amino acid, organic acid and sugar phosphate determination in a single run.
- Metabolism of U-^{13}C glucose and 1-^{13}C acetate in ZR-75-1 cells studied.

GRAPHICAL ABSTRACT



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ABSTRACT

A high performance liquid chromatography (HPLC) tandem mass spectrometric (MS/MS) method has been developed for the simultaneous determination of fifteen glucose, or acetate derived metabolites isolated from tumor cells. Glycolytic and tricarboxylic acid (TCA) cycle metabolites as well as acidic amino acids were separated on a HPLC porous graphitic carbon (PGC) column and simultaneously determined by means of triple quadrupole MS/MS using multiple reaction monitoring (MRM). Target compounds were eluted within 10 min with 8% v/v formic acid as an electronic modifier added to a 4:1 v/v methanol water mobile phase. The calibration is linear in the 1–100 μM concentration range for each analyte. The limit of detection ranges between 0.39 and 2.78 μM for the analytes concerned. To test the PGC–HPLC–MS/MS method in metabolomic studies, ZR-75.1 human mammary adenocarcinoma cells were labeled with U-^{13}C glucose or 1-^{13}C acetate. Applying the MRM mode, the incorporation of ^{13}C into metabolites, isolated from the tumor cells, and derived from glucose or acetate, could be properly identified.
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1. Introduction

Numerous biological studies on tumors have provided evidence for the elevated glycolysis and impaired tricarboxylic acid (TCA) cycle [1], thus there is a paramount interest to introduce appropriate bioenergetic assays in clinical oncology. These target

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compounds are highly polar. Sometimes, they have very similar chemical structure and poor UV absorption. These metabolites can be described as simple organic acids, amino, dicarboxylic-, tricarboxylic-, and phosphocarboxylic acids, aldehyde and sugar phosphates, and sugar diphosphates. Furthermore, the above-mentioned analytes can be found at low concentrations in tissues or cell cultures. Consequently, combined separation and analytical methods exhibiting the reliable determination of the metabolites in the biological samples is required.

Liquid chromatography–mass spectrometry (LC–MS) has a clear advantage for this type of analytical task [2]. The MS allows detection of co-eluting components. Moreover, it provides sufficient resolution and sensitivity for the detection of stable isotope labeled molecules at low concentrations, with high sample throughput. Several LC–MS methods have been reported for the determination of TCA intermediates and/or sugar phosphates. Generally, these methods are based on five different chromatographic principles: (i) hydrophilic interaction liquid chromatography (HILIC); (ii) ion exchange chromatography; (iii) ion pair chromatography; (iv) modified reversed phase chromatography and (v) combined/mixed stationary phase chromatography [3–13]. The use of polar embedded stationary phases is limited because of the poor retention of the above-mentioned compounds [8], while the mixed stationary phases are still under development.

Up to now, the best separation of energy metabolites has been achieved on C18 stationary phases by using ion pairing reagents (octyl-, hexyl- and tributylamine) [5,9–12]. The main advantage of this method is the adequate separation and simultaneous determination of several compounds with similar chemical structure. However, the use of ion pairing reagents is practically incompatible with MS systems, because they cause memory effects, when electrospray ionization is applied in positive mode. Common ion pairing reagents such as tributylamine do not cause interference in negative ionization mode, but this implies high-cost analyses as two LC–MS methods should be applied, namely, one in positive and one in negative ionization mode in order to cover the determination of all compounds of interests. By comparing chromatographic separation for polar metabolites applying tributylamine as ion pairing reagent with a HILIC one, it turned out that the ion pairing chromatographic method was better in terms of sensitivity and resolution [12,13]. Porous graphitic carbon (PGC) columns were successfully applied for the separation of polar compounds [14,15]. Separation of some of these molecules on PGCs has also been reported. For example, Antonio et al. reported the simultaneous determination of carbohydrates and carbohydrate phosphates on the PGC stationary phase [16]. Separation of these analytes was achieved in two steps by gradient elution using acetonitrile (ACN) and formic acid (FA) for carbohydrates and carbohydrate phosphates, respectively [16].

The aim of this study was to develop a robust PGC–LC–MS/MS method for the determination of a certain set of metabolites involved in bioenergetic pathways such as glycolysis and TCA cycle by using common chromatographic eluents. Beyond doubt, the various chemical properties of metabolites represent a great challenge in their identification in cell cultures.

2. Materials and methods

2.1. Chemicals and reagents

Throughout the experiments, deionized Milli-Q water with a resistivity of 18 MΩ cm was used. All chemicals and reagents were of analytical grade if not indicated otherwise. Standards of alpha-ketoglutaric acid (AKG), L-alanine (ALA), L-aspartic acid (ASP), citric acid (CIT), dihydroxyacetone phosphate (DHAP), fructose 6-phosphate (F6P), fructose 1,6-bisphosphate (FBP), fumaric acid (FUM), glucose 6-phosphate (G6P), L-glutamic acid (GLU), 3-phosphoglyceric acid (3 PG), L-lactic acid (LAC), L-malic acid (MAL), phosphoenolpyruvic acid (PEP), pyruvic acid (PYR), ribose-5-phosphate (R5P), succinic acid (SUC) were purchased from Sigma–Aldrich (Budapest, Hungary).

Fetal bovine serum, DMEM and D5030 (deficient in glucose, PYR, glutamine) media were purchased from Sigma–Aldrich (Budapest, Hungary). Cell culture plates were produced by Sarstedt (Budapest, Hungary).

Stable isotopes of U-^{13}C glucose and 1-^{13}C acetate were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Phosphate buffered saline (PBS) was also purchased from Sigma–Aldrich (Budapest, Hungary).

2.2. Instrumentation

Waters Acquity LC system was used with a 150 mm × 1 mm Hypercarb column (Thermo Fisher Scientific Inc., Waltham MA, USA). The flow rate was set to 50 μL min^{-1}. Binary solvents were used for gradient elution. The solvent A consisted of 80% v/v methanol (MeOH) and 20% v/v H_2O, and the solvent B consisted of 70% v/v MeOH, 22% v/v H_2O and 8% v/v FA. The initial composition of the eluent was 100% A for 2 min followed by a linear gradient to 100% B in 14 min. Eluent composition was then set again to 100% A in 1 min and the column was further washed with 100% A for 20 min. For each measurement, a sample aliquot of 5–10 μL was injected onto the column.

For detection, a Waters Micromass Quattro Micro triple quadrupole mass spectrometer (Waters Corporation, Milford MA, USA) was used operating with an electrospray source in the negative ion mode. Nitrogen was used as a sheath and collision gas as well. The electrospray source settings were as follows: sprayer capillary voltage 2.9 kV, cone voltage 25 V, source temperature 120 °C, desolvation temperature 200 °C, sheath (cone) gas flow 50 L h^{-1}, desolvation gas flow 800 L h^{-1}.

The analytes were detected in multiple reaction monitoring (MRM) mode. The selected masses and collision energies can be found in Table 1. Optimum parameters for MRM experiments were determined using a standard mixture consisting of 100 μM of each analyte, in 4:1 v/v MeOH–water, delivered with a syringe pump at a flow rate of 5 μL min^{-1}. For sensitivity check, FA, trifluoroacetic acid (TFA) solutions as well as a 1:1 v/v mixture of ACN–iso-propanol (iPRO) were used as a solvent for the target compounds. Data acquisition and evaluation were conducted on a personal computer using Masslynx 4.1 (Waters Corporation, Milford, MA, USA) software.

In order to check the accuracy of the method, gas chromatography–mass-spectrometry (GC–MS) was applied according to Koek et al. [17]. Briefly, a QP2010 ULTRA GC–MS instrument supplied by Shimadzu (Tokyo, Japan) was used in split or splitless mode. Separation of metabolites was achieved on a SLB-5ms fused silica 30 m × 0.25 mm × 0.1 μm GC column (Supelco, Bellefonte, PA, USA).

2.3. Cell culture and sample preparation

Human tumor cell line ZR-75.1 (mammary adenocarcinoma) (CRL-1500) was purchased from ATCC (LGC Standards GmbH, Wesel, Germany). Cells were cultured at the 1st Institute of Pathology and Cancer Research, Budapest, Hungary. In order to prepare subconfluent cultures, cells were plated and cultured to 80% confluency in cell culture flasks of 250 mL. These cells were grown in 10 mL of RPMI1640 medium supplemented with 10% v/v fetal bovine serum, 100 IU penicillin and 50 μg mL^{-1} streptomycin. After culturing for 48 h at 37 °C in a humidified 5% v/v CO_2 atmosphere, the culture medium was removed, then cells were washed twice

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