Journal of Chromatography B 1060 (2017) 76–83

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

Ultra-performance liquid chromatography-tandem mass spectrometry-based multiplex enzyme assay for six enzymes associated with hereditary hemolytic anemia

Chul Min Park[a], Kyunghoon Lee[b,c], Sun-Hee Jun[c], Sang Hoon Song[b,d], Junghan Song[b,c,*]

[a] Department of Laboratory Medicine, Dongnam Institutes of Radiology and Medical Sciences, Busan, South Korea
[b] Department of Laboratory Medicine, Seoul National University College of Medicine, Seoul, South Korea
[c] Department of Laboratory Medicine, Seoul National University Bundang Hospital, Seongnam-si, South Korea
[d] Department of Laboratory Medicine, Seoul National University Hospital, Seoul, South Korea

ARTICLE INFO

Keywords:
Hemolytic anemia
Enzyme assay
Glucose-6-phosphate dehydrogenase
Pyruvate kinase
Pyrimidine 5′-nucleotidase
Mass spectrometry

ABSTRACT

Deficiencies in erythrocyte metabolic enzymes are associated with hereditary hemolytic anemia. Here, we report the development of a novel multiplex enzyme assay for six major enzymes, namely glucose-6-phosphate dehydrogenase, pyruvate kinase, pyrimidine 5′-nucleotidase, hexokinase, triosephosphate isomerase, and adenosine deaminase, deficiencies in which are implicated in erythrocyte enzymopathies. To overcome the drawbacks of traditional spectrophotometric enzyme assays, the present assay was based on ultra-performance liquid chromatography-tandem mass spectrometry (UPLC–MS/MS). The products of the six enzymes were directly measured by using ion pairing UPLC–MS/MS, and the precision, linearity, ion suppression, optimal sample amounts, and incubation times were evaluated. Eighty-three normal individuals and 13 patients with suspected enzymopathy were analyzed. The UPLC running time was within 5 min. No ion suppression was observed at the retention time for the products or internal standards. We selected an optimal dilution factor and incubation time for each enzyme system. The intra- and inter-assay imprecision values (CVs) were 2.5–12.1% and 2.9–14.3%, respectively. The linearity of each system was good, with R^2 values > 0.97. Patient samples showed consistently lower enzyme activities than those from normal individuals. The present ion paring UPLC–MS/MS assay enables facile and reproducible multiplex evaluation of the activity of enzymes implicated in enzymopathy-associated hemolytic anemia.

1. Introduction

Hereditary hemolytic anemias are collectively defined as hemolytic anemia of genetic origin. Based on the underlying pathological cause, these may be divided into abnormalities of the erythrocyte membrane, hemoglobin, or metabolic enzymes [1]. The abnormalities of erythrocyte metabolic enzymes, termed erythrocyte enzymopathies, are associated with normocytic normochromic anemia and common signs of hemolysis such as increased plasma level of bilirubin and lactate dehydrogenase, low levels of haptoglobin, and high reticulocyte count [2].

The enzymes commonly implicated in hemolytic anemia are glucose-6-phosphate dehydrogenase (G6PD), pyruvate kinase (PK), and pyrimidine 5′-nucleotidase (P5′N). Approximately 2.9% of the world's population is genetically G6PD-deficient, with over 40 million reported cases [3]. Other enzymes involved in erythrocyte metabolism, such as hexokinase (HK), triosephosphate isomerase (TPI), and adenosine deaminase (AD), are also implicated in the pathogenesis of hemolytic anemia [4].

Classical methods, such as those described by Beutler, are most commonly used for the diagnosis of red blood cell enzymopathies. Specific reactions are set up for each enzyme based on spectrophotometric determination of respective enzyme activities, and changes in cofactor substrates (NAD+, NADP+, NADH, or NADPH) are monitored at 340 nm for a set duration [5]. However, these spectrophotometry-based assays generally require multiple enzyme reaction

Abbreviations: UPLC–MS/MS, ultra-performance liquid chromatography-tandem mass spectrometry; G6PD, glucose-6-phosphate dehydrogenase; PK, pyruvate kinase; P5′N, pyrimidine 5′-nucleotidase; HK, hexokinase; TPI, triosephosphate isomerase; AD, adenosine deaminase; MRM, multiple reaction monitoring; CAP PT, College of American Pathologists proficiency testing

* Corresponding author at: Department of Laboratory Medicine, Seoul National University Bundang Hospital, 82, Gumi-ro 173 beon-gil, Bundang-gu, Seongnam-si, Gyeonggi-do 13620, Korea.
E-mail address: songjhcp@snu.ac.kr (J. Song).

http://dx.doi.org/10.1016/j.jchromb.2017.05.040
Received 4 October 2016; Received in revised form 30 May 2017; Accepted 31 May 2017
Available online 03 June 2017
1570-0232/

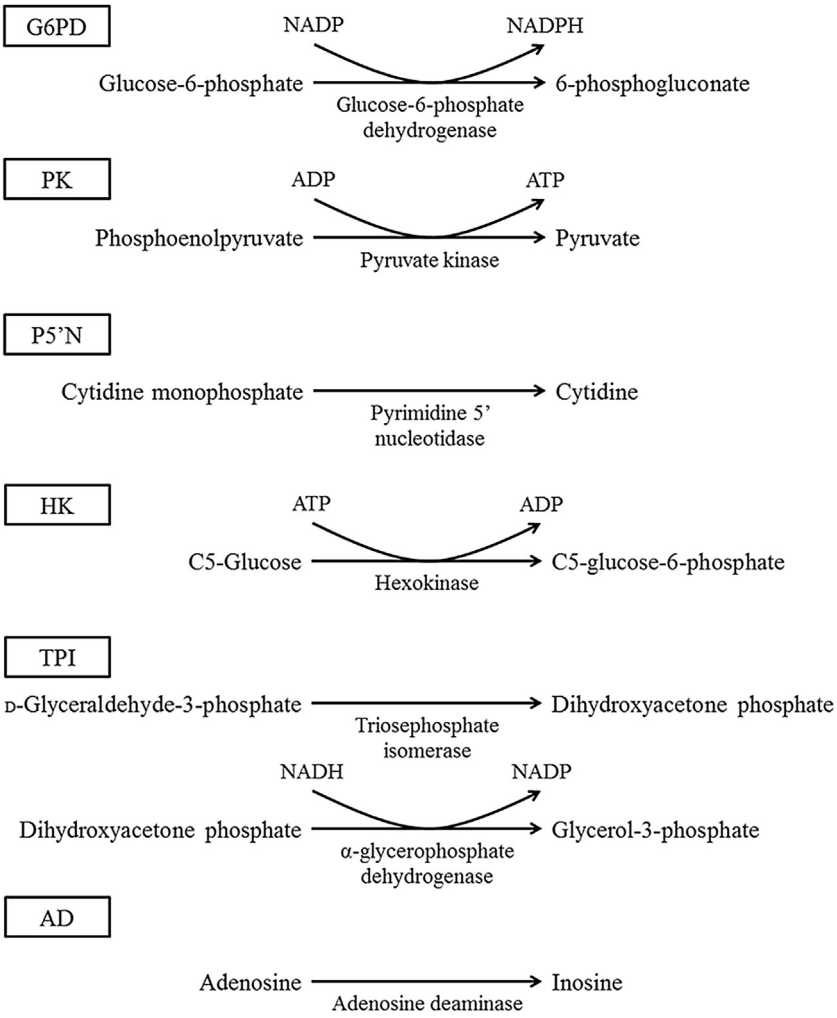

Fig. 1. Reaction scheme for six erythrocyte enzymes. Abbreviations: G6PDglucose-6-phosphate dehydrogenase; PKpyruvate kinase; P5′Npyrimidine 5′ nucleotidase; HKhexokinase; TPItriosephosphate isomerase; ADadenosine deaminase.

Table 1
Reaction mixtures and ingredients for enzyme reactions.

Set	Enzyme	Substrate	Erythrocytes	Buffer	Additional reagents	Internal standards
1	G6PD	200 μL 20 mM G6P	20 μL	50 μL buffer1[a]	200 μL 10 mM NADP	20 μL 0.2 mM C1-Gal-1-P
1	PK	100 μL 25 mM PEP	20 μL	100 μL buffer1[a]	100 μL 1 M KCl 50 μL 30 mM ADP	20 μL 0.2 mM C1-Gal-1-P
1	P5′N	100 μL 4.6 mM CMP	20 μL	50 μL buffer1[a]		20 μL 0.5 mg/mL C5-cytidine
2	HK	200 μL 10 mM C5-glucose	20 μL	100 μL buffer1[a]	400 μL 20 mM ATP	20 μL 0.2 mM C1-Gal-1-P
2	TPI	20 μL 5 mM D-GAP	20 μL	100 μL buffer2[b]	100 μL NADH 50 μL α-glycerophosphate dehydrogenase	20 μL 0.2 mM C1-Gal-1-P
2	AD	100 μL 2 mM adenosine	20 μL	100 μL buffer2[b]		20 μL 1 mg/mL C5-inosine

Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; PK, pyruvate kinase; P5′N, pyrimidine 5′ nucleotidase; HK, hexokinase; TPI, triosephosphate isomerase; AD, adenosine deaminase; G6P, glucose-6-phosphate; PEP, phosphoenolpyruvate; CMP, cytidine monophosphate; D-GAP, D-glyceraldehyde-3-phosphate; NADP, nicotinamide adenine dinucleotide phosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; C1-Gal-1-P, C1-galactose-1-phosphate.

[a] buffer1: 1 M Tris-HCl with 5 mM EDTA and 0.1 M $MgCl_2$, pH 8.0.

[b] buffer2: 1 M Tris-HCl with 5 mM EDTA, pH 8.0.

steps and separate measurement of enzyme activities, which is time-consuming and labor-intensive. To overcome these disadvantages, multiplex enzyme assays utilizing mass spectrometry were first developed for lysosomal storage disorders [6,7]. Here, we have developed liquid chromatography-tandem mass spectrometry (LC–MS/MS) assays for three galactosemic enzymes [8]. However, to the best of our knowledge, there are no reports on LC–MS/MS assays for RBC enzymes associated with RBC enzymopathies.

In the present work, we developed an ion-paring ultra-performance liquid chromatography-tandem mass spectrometry (UPLC–MS/MS)-based novel multiplex enzyme assay for larger number of enzymes including G6PD, PK, P5′N, HK, TPI, and AD, deficiencies of which are implicated in erythrocyte enzymopathies.

2. Materials and methods

2.1. Chemicals and reagents

HPLC-grade methanol (Avantor Performance Materials, Inc., Center Valley, PA, USA) and water were used. α-D-[UL-$^{13}C_6$]glucopyranosyl 1-phosphate (C6-glucose-1-phosphate), α-D-[1-^{13}C]glucopyranosyl 1-phosphate (C1-glucose-1-phosphate), D-[2,3,4,5,6-$^{13}C_5$]glucose (C5-glucose),