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Hydrophilic-interaction liquid chromatography–tandem mass spectrometric determination of erythrocyte 5-phosphoribosyl 1-pyrophosphate in patients with hypoxanthine–guanine phosphoribosyltransferase deficiency

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ABSTRACT

Mutations in the gene encoding hypoxanthine–guanine phosphoribosyltransferase (HPRT) cause Lesch–Nyhan disease (LND) and its variants (LNV). Due to the technical problems for measuring the HPRT activity in vitro, discordances between the residual HPRT activity and the clinical severity were found. 5-Phosphoribosyl 1-pyrophosphate (PRPP) is a substrate for HPRT. Since increased PRPP concentrations were observed in erythrocytes from patients with LND and LNV, we have turned our attention to erythrocyte PRPP as a biomarker for the phenotype classification. In the present work, a method for determination of PRPP concentration in erythrocyte was developed using liquid chromatography–tandem mass spectrometry (LC–MS/MS) with multiple reaction monitoring (MRM). Packed erythrocyte samples were deproteinized by heating and the supernatants were injected into the LC–MS/MS system. All measurement results showed good precision with RSD <6%. PRPP concentrations of nine normal male subjects, four male patents with LND and six male patients with LNV were compared. The PRPP concentrations in erythrocyte from patients with LND were markedly increased compared with those from normal subjects, and those from patients with LNV were also increased but the degree was smaller than those with LND. The increase pattern of PRPP concentration in erythrocyte from patients with HPRT deficiency was consistent with the respective phenotypes and was correlated with the disease severity. PRPP concentration was suggested to give us supportive information for the diagnosis and the phenotype classification of LND and LNV.

1. Introduction

Hypoxanthine–guanine phosphoribosyltransferase (HPRT, EC.2.4.2.8) is a purine salvage enzyme that catalyzes the condensation of 5-phosphoribosyl 1-pyrophosphate (PRPP) and hypoxanthine or guanine to yield inosine 5-monophosphate (IMP)

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and guanosine 5-monophosphate, respectively. The mutations of the gene encoding HPRT (*HPRT1*) have high degree of heterogeneity. More than 600 different *HPRT1* mutations are currently known [1]. A complete deficiency of HPRT activity leads to Lesch–Nyhan disease (LND), which is characterized by uric acid overproduction, motor dysfunction, cognitive disability, and self-injurious behavior [2]. Lesch–Nyhan variant (LNV) resulted from a partial deficiency of HPRT activity is characterized by hyperuricemia and variable degree of neurological manifestations, depending on the residual enzyme activity [3].

HPRT activity is assayed for diagnosis of LND and LNV, and is measured with erythrocyte lysates [4,5] or the intact cells such as fibroblasts [5,6] and lymphocytes [7]. Although it is considered that the residual activity of mutational HPRT correlates with clinical severity [4,6], there are several exceptions that are not consistent with this rule [5,8–10]. When the mutational HPRT is unstable outside of the normal cellular milieu, in vitro assay is prone to give artificially low residual activity [1,11]. Another mechanism leading to reduced HPRT function involves altered affinities toward hypoxanthine, guanine or PRPP. For such mutants, in vitro assays employing high substrate concentration may yield high apparent activities, while physiological concentrations of substrates available in vivo may result in little or no actual activity [1,11,12]. These observations emphasize the importance of other supportive biomarker for the diagnosis and the phenotype classification of LND and LNV.

PRPP is an essential compound for the de novo pathway of purine synthesis as well as the synthesis of pyrimidine compounds. Since PRPP concentrations in erythrocyte are markedly increased in patients with HPRT deficiency [13], we have focused on the availability of PRPP as the biomarker. A number of enzymatic assays have been described for the measurement of PRPP in biological fluids [14–18]. The basic approach utilizes the conversion of PRPP to a radioactive derivative through its reaction with a ^{3}H- or ^{14}C-labeled base under catalysis by the appropriate phosphoribosyltransferase. The methods have involved trapping of evolved $^{14}CO_2$ or separation of radioactive products by various chromatographic techniques. These radiochemical methods have required special equipment and several complicated procedures. As a non-radioisotope method, IMP formation in lymphoblastoid cell from patients with LND and LNV was measured by liquid chromatography–mass spectrometry (LC–MS) [19].

Because PRPP has no chromophore groups and the 1-pyrophosphate group and 5-phosphate group of PRPP are unstable under derivatization conditions, there are no reports for direct determination of PRPP in biological fluids by chromatographic procedures. Since LC coupled with tandem mass spectrometry (LC–MS/MS) is an analytical technique with high selectivity, sensitivity and reproducibility and does not require derivatization, the technique would be suitable for the measurements of the relatively unstable compounds, such as PRPP.

The objectives of the present paper are to develop an LC–MS/MS method for the direct determination of PRPP in erythrocyte and to investigate the availability of erythrocyte PRPP concentration as a supportive biomarker for the diagnosis and the phenotype classification of LND and LNV.

2. Materials and methods

2.1. Materials

PRPP tetrasodium salt (purity, 81%) was purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile and 25% aqueous ammonia were purchased from Wako Pure Chemicals (Osaka, Japan). Deionized water (18 MΩ cm, Millipore, Bedford, MA, USA) was used for sample preparation and the HPLC mobile phase. All other chemicals and solvents were of analytical reagent grade and used without further purifications.

2.2. Stock solutions

A stock solution of 100 μM PRPP in 50 mM Tris–HCl buffer (pH 7.0) was prepared. Working solutions with PRPP concentration in the range of 0.5–50 μM were prepared by diluting the stock solution with 50 mM Tris–HCl buffer (pH 7.0). Storage of these solutions at 4 °C did not result in any detectable decomposition for at least one week.

2.3. Subjects

This study was approved by the ethics committee of the Tokyo University of Pharmacy and Life Sciences, and written informed consent was obtained from all individuals. Nine healthy male subjects, four male patients with LND (patient AA, BB, CC and DD) and six male patients with LNV [patient EE, FF, GG-a, GG-b, GG-c (siblings) and HH] were recruited. We have previously reported the mutations of *HPRT1* and the activities of HPRT in erythrocytes in the enrolled patients [20–22] except patient CC.

2.4. Sample preparation

Blood was collected in heparinized tubes. Erythrocytes were isolated by centrifugation at 5000 × *g* for 10 min at 4 °C and washed twice with saline and lysates were obtained by freezing and thawing the red cells twice. The samples were stored at −80 °C until analysis.

The lysate samples were thawed in 4 °C water bath. Known amounts of PRPP in the range of 0–250 pmol dissolved in 20 μl of 50 mM Tris–HCl buffer (pH 7.0) were spiked to 20 μl of thawed lysate samples and then the samples were diluted with 50 mM Tris–HCl buffer (pH 7.0) to a volume of 200 μl. After heating for 45 s in boiling water, the samples were immediately chilled on ice. During this step all interfering enzyme activities were destroyed while PRPP remained intact. After centrifugation at 5000 × *g* for 10 min at 4 °C, the supernatant was filtered through a GHP Nanosep membrane filter (hydrophilic polypropylene, 0.45 μm; Pall Life Sciences, Ann Arbor, MI, USA). Each filtrate was transferred to an auto-sampler vial and 5–10 μl of the filtrate was subjected into LC–MS/MS.

2.5. Chromatography and mass spectrometry

The HPLC device comprised an Agilent (Santa Clara, CA, USA) Liquid Chromatograph 1200SL Series equipped a binary pump, an online vacuum degasser, an automatic liquid sampler, a thermostatted column compartment and a diode-array detector. Separation was achieved using Luna Amino-column (50 mm × 2.0 mm ID, 3 μm; Phenomenex, Santa Barbara, USA). The column temperature was set at 25 °C. Mobile phases were a solution of 0.025% ammonia in water and acetonitrile (95:5, v/v; mobile phase A) and 100% acetonitrile (mobile phase B). The gradient elution was as follows: initial 15% A and 85% B; linear gradient to 100% A in 10 min; hold for 5 min, returned to initial conditions in 1 min, followed by equilibration for 10 min. The flow rate was 300 μl/min. Run-to-run time was 26 min.

Mass spectrometry analyses were performed Agilent 6410 triple quadrupole tandem mass spectrometer equipped with an electrospray source. The operating conditions for the electrospray ionization (ESI) interface were as follows: a source temperature of 300 °C, a nebulizer gas of 30 psi and a desolvation gas of 8 l/min.