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Development and validation of a 2nd tier test for identification of purine nucleoside phosphorylase deficiency patients during expanded newborn screening by liquid chromatography-tandem mass spectrometry

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Abstract

Background: Purine nucleoside phosphorylase (PNP) deficiency has been recently introduced in the newborn screening program in Tuscany. In order to improve the PNP screening efficiency, we developed a 2nd tier test to quantify PNP primary markers deoxyguanosine (dGuo) and deoxyinosine (dIno).
Methods: Dried blood spots (DBS) samples were extracted with 200 μL of methanol and 100 μL of water (by two steps). Internal standards were added at a final concentration of 10 μmol/L. After extraction, samples were analysed by LC-MS/MS. The chromatographic run was performed in gradient mode by using a Synergi Fusion column.
Results: The assay was linear over a concentration range of 0.05–50 μmol/L (R^2>0.999) for dGuo and 0.5–50 μmol/L (R^2>0.998) for dIno. Intra- and interassay imprecision (mean CVs) for dIno and dGuo ranged from 2.9% to 12%. Limit of quantitaion (LOQ) were found to be 0.05 μmol/L and 0.5 μmol/L for dGuo and dIno, respectively. The reference ranges, obtained by measuring dGuo and dIno concentrations on DBS, were close to zero for both biomarkers. Moreover, DBS samples from seven patients with confirmed PNP were retrospectively evaluated and correctly identified.
Conclusions: The LC-MS/MS method can reliably measure dIno and dGuo in DBS for the diagnosis of PNP. Validation data confirm the present method is characterised by good reproducibility, accuracy and imprecision for the quantitation of dIno and dGuo. The assay also appears suitable for use in monitoring treatment of PNP patients.

Keywords: 2nd tier test; dried blood spot; newborn screening; purine nucleoside phosporilase; purine nucleoside phosphorylase deficiency; severe combined immunodeficiency; tandem mass spectrometry.

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Introduction

Purine nucleoside phosphorylase (PNP) deficiency represents approximately the 4% of severe combined immunodeficiencies (SCID) [1]. PNP is a key enzyme in the purine degradation and salvage pathway that catalyses phosphorilation of inosine-deoxyinosine and guanosine-deoxyguanosine to hypoxantine and guanine. Then, metabolites are converted to uric acid or back to

nucleotides through the salvage pathway [2]. The enzyme deficiency results in a particularly serious accumulation of all four metabolites but most of all deoxyinosine and deoxyguanosine. The phosphorylation of deoxyguanosine leads to the formation of a lymphocyte toxic metabolite, the deoxyguanosine triphosphate (dGTP) [3]. dGTP causes inhibition of ribonucleotide reductase and DNA synthesis [4]. As a result, uric acid levels should be low and PNP deficiency may be suspected in the presence of hypouricaemia. Nevertheless, recent cases described many patients with normal plasma levels of uric acid and then this marker should not be used in differential diagnosis of PNP deficiency [5, 6].

Typically PNP deficiency causes T-cell immunodeficiency and for this reason patients suffer from recurrent infections of the respiratory tract, neurologic abnormalities and autoimmunity [1, 5, 7–9].

Early detection of primary immunodeficiency is recognized as important for avoiding infectious complications that compromise outcomes [10].

Some newborn screening (NBS) programs evaluate T-cell receptor excision circle (TREC) and kappa-deleting recombination excision circle (KREC) levels on dried blood spots (DBSs) by quantitative real-time PCR. However, Azzari et al. demonstrated that both, TREC and KREC quantitative analysis, might fail in identification of some forms of delayed or late-onset immunodeficiencies [11].

In 2011, we developed a method by tandem mass spectrometry (MS/MS) to include adenosine deaminase deficiency (ADA-SCID) in the NBS for inborn errors of metabolism [12, 13]. This inexpensive and sensitive procedure, applied to NBS, allowed to identify an infant with ADA deficiency in pre-symptomatic stage of illness to prevent progression to severe complications [13].

Recently, we have also included PNP metabolites in the NBS panel demonstrating that MS/MS is able to identify PNP patients [14].

Since patients are characterized by a profile of elevated metabolite levels (Guo, Ino, dGuo and dIno) the false-positive rate is very low. However, it is difficult to exclude possible contaminants in flow injection analysis used for NBS purposes and a 2nd tier test is an useful tool to avoid parental anxiety due to an unnecessary recall testing. The 2nd tier test is performed when first NBS result is positive in order to detect the disease marker by chromatographic separation coupled to MS/MS under the optimum operating condition [15].

In this work, we present a validated method able to detect and quantify the primary markers of PNP deficiency (dGuo and dIno). It is not only suitable as 2nd tier test but also it is a rapid, noninvasive diagnostic test when a PNP deficiency is suspected.

Materials and methods

Chemicals and reagents

[$^2H_2$] 2′-deoxyguanosine monohydrate and [$^{15}N_4$]-deoxyinosine were purchased from Omicron Biochemicals (South Bend, IN, USA). Stock solutions of labelled standards were prepared at 10 mmol/L in HPLC grade water and stored at –20 °C. Working solutions were daily prepared by dilution to a final concentration of 10 μmol/L from stock solutions.

dGuo and dIno reference standards to prepare calibration curve and quality controls (QC) were purchased from Sigma Aldrich (St. Louis, MO, USA).

All solvents were HPLC grade purity and were purchased from Sigma Aldrich (St. Louis, MO, USA). Blood was spotted on filter paper grade 903 (Whatman GmbH, Dasel, Germany).

Samples

Blood spot specimens were collected from 250 healthy controls (150 newborns and 100 adults) and anonymously analysed in order to establish reference ranges. We retrospectively analyzed stored DBS from seven patients with confirmed PNP deficiency. One of samples was collected from a newborn on 3rd day of life. All samples were collected at time of diagnosis before any treatment has been started. Informed consent were obtained from patients before sample analysis.

All experiments were conducted in compliance with institutional review board guidelines (regional pilot project protocol no. 7949/2011). DBS were stored in sealed plastic bags at –20 °C until analysis.

Sample preparation

A 3.2 mm disk was punched from DBS and extracted with 200 μL of methanol containing 10 μmol/L of IS. A volume of 100 μL of water was added in a second step to prevent blood haemolysis and to facilitate purine extraction from paper. The mixture was incubated by a gentle shaking for 25 min at 37 °C. Samples were transferred to a clean micro-well plate and analysed.

Preparation of calibration standards and quality control (QC) samples

Calibration standards and QC samples were prepared by spotting 25 μL of pooled whole blood on filter paper. After extraction, different concentrations of dGuo and dIno were added to the extracts. QC samples for imprecision studies were prepared at 0.1 μmol/L (LQC), 10 μmol/L (MQC), 25 μmol/L (HQC) for dGuo and at 1 μmol/L (LQC), 10 μmol/L (MQC), 25 μmol/L (HQC) for dIno. For evaluation of the linearity the calibration curve was constructed at concentrations of 0.1, 1, 5, 10, 12.5, 25, 50 μmol/L for dGuo and 0.5, 1, 5, 10, 12.5, 25, 50 μmol/L for dIno. For the validation study QC samples and calibration standards were freshly prepared.

LC-MS/MS

Analyses were performed using Infinity 1260 System (Agilent Technologies, Waldbronn, Germany), coupled with an API 4000 triple quadrupole mass spectrometer (AB Sciex, Toronto, Canada) equipped with a TurboV-Spray® source with the turbo gas temperature set at 425 °C. The source operates in positive ionization polarity at a potential of +5500 V. Mass parameters are listed in Table 1. Each instrument module was fully controlled by the Analyst Software (Version 1.5.2).

The chromatographic run was performed by using a Synergi Fusion column, 4 µm, 150×2 mm (Phenomenex, Torrance, CA, USA) at a flow rate of 250 µL/min; the eluate was flowed directly into the ESI source without splitting. The mobile phase, composed of a mixture of 0.25% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), was applied in a gradient mode. The gradient started with 10% of solvent B for 0.1 min, ramped up to 35% in 4 min, then was increased to 80% in 0.1 min. After 1 min the initial condition was restored and held for 5 min. Injection volume was 3 µL. The analyte concentrations were calculated using the option Quantitate of the Analyst Software (AB Sciex, Toronto, Canada).

Method validation

This method was validated in terms of linearity, specifity, intra- and inter-day accuracy and imprecision.

The lower limit of quantitation (LLOQ) was determined preparing a specific calibration curve in the range 0–1 µmol/L for both analytes; the residual standard deviation of the regression line and its slope were used to calculate LLOQ, according to ICH guideline [16].

The accuracy and imprecision were determined by the analysis of QC samples at three concentrations. Ten replicates of each level of QC samples were assayed in one run for the intraday accuracy and imprecision evaluation. For the interday experiment, three replicates of each QC sample were analysed within ten different days. The intra- and interday imprecision and accuracy of the assay were expressed as percent coefficient of variation (CV) and percent bias values, respectively.

In order to evaluate the matrix effect on the ionization of the analytes, blank DBS samples were extracted and spiked at LQC, MQC and HQC concentrations for both analytes and compared to solvent samples spiked at the same concentrations. All samples contained 10 µmol/L of IS.

Table 1: Summary of analytes and their MRM transitions and MS parameters. CXP and EP values were 22 V and 10 v, respectively, for all transitions.

Analytes	Q1, *m/z*	Q3, *m/z*	DP, V	CE, V
dGuo	268.2	152.1	40	18
dIno	253.2	137.1	30	13
dGuo IS	270.2	152.1	40	18
dIno IS	257.2	141.1	30	13

CXP, Collision exit potential; EP, entrance potential; DP, declustering potential; CE, collision energy.

Results and discussion

PNP deficiency results in accumulation of purines (Guo, Ino, dIno and dGuo), generated from DNA degradation. In particular, the deoxynucleoside accumulation are toxic to lymphocytes, leading to profound T cell-mediated immunosuppression. Under normal physiological conditions, the deoxynucleosides, unlike Guo and Ino, are undetectable (dGuo) or close to zero (dIno) on human whole blood because they are rapidly phosphorylated by PNP. This makes dGuo and dIno the best biomarker candidates to identify PNP deficiency by MS/MS (Figure 1) although increased levels of all the four purines were found in the samples from PNP patients retrospectively analysed [14].

Purine nucleoside phosphorylase is a cytoplasmic enzyme that is present in all mammalian cells. For this reason, QC samples cannot be prepared by directly adding purine reference standards to the whole blood due to the rapid enzymatic metabolism, as previously reported [13].

The chromatographic conditions were optimized in order to obtain a good resolution of peaks within shorter time. The resolutions of these compounds were compared with different reversed phase analytical columns and the Synergi Fusion column (4 µm, 150×2 mm) was selected since results showed that only this column could supply appropriate resolution and response. The choice of mobile phase is equally important. In this study, acetonitrile-water was chosen because this combination gave the best resolution for the analytes. No carry over of peaks were observed at the retention time of dGuo and dIno. The chromatographic column showed robust performances even after a several hundreds of injections.

A calibration curve with low concentrations (<1 µmol/L) was generated for dIno and dGuo to determine the LLOQ by using the following equation: LLOQ=10 σ/S, where σ=residual standard deviation and S=the slope of the regression line. LLOQ were found to be 0.05 µmol/L and 0.5 µmol/L for dGuo and dIno respectively.

The method was linear from 0.05 to 50 µmol/L (R^2>0.999) for dGuo and 0.5 to 50 µmol/L (R^2>0.998) for dIno.

The mean intraday and interday CVs for the analytes of interest are reported in Table 2. These data confirm that the present method has proven to have good reproducibility, accuracy and imprecision for the quantisation of dIno and dGuo.

Matrix effect could affect the reproducibility from the analyte or the internal standard of the assay and was calculated comparing peak areas from spiked analytes in DBS extracted matrix versus pure standards prepared in

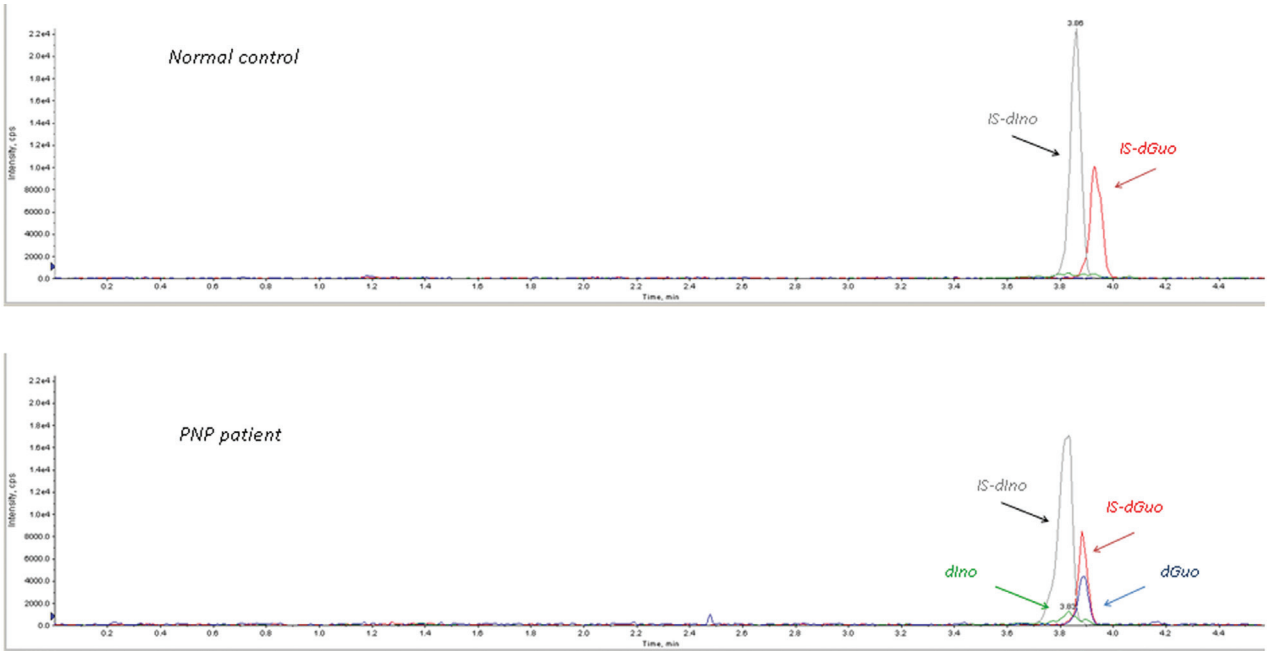

Figure 1: Extracted ion chromatogram from a normal control and a PNP patient.

Table 2: Intra- and interday precision and accuracy of the assay.

Intraday (10 replicates)					Interday (In triplicate for 10 days)				
Expected concentration, µmol/L	Mean, µmol/L	SD	CV, %	Accuracy (% Bias)	Expected concentration, µmol/L	Mean, µmol/L	SD	CV, %	Accuracy (% Bias)
Deoxyguanosine					Deoxyguanosine				
0.1	0.11	0.01	7.9	+10.0	0.1	0.11	0.01	5.19	+10.0
10	10.09	0.66	6.5	+0.9	10	10.48	0.44	4.2	+4.8
25	23.95	1.48	6.1	−4.2	25	24.09	1.02	4.22	−3.7
Deoxyinosine					Deoxyinosine				
1	0.98	0.12	12.0	−2.0	1	1.01	0.08	7.8	+0.6
10	10.27	0.84	8.2	+2.7	10	10.30	0.30	2.9	+3.0
25	24.50	0.84	3.4	−2.0	25	24.45	0.77	3.2	−2.2

solvent. No matrix effect was observed in any of the three QC concentrations for both analytes.

Concentration levels to establish reference range on DBS from a healthy population resulted be undetectable for dGuo and less than the LLOQ (<0.5 µmol/L) for dIno. No significant differences were observed between newborn and adult ranges.

We previously reported a LC-MS/MS method to include PNP-SCID in expanded newborn screening panel. DBS samples from 9 patients with diagnosed PNP-SCID were retrospectively analysed by using LC/MS-MS analysis in the flow-injection mode (FIA) as reported by la Marca et al. [14]. Unfortunately, we had sufficient material only of 7 patients to carry out 2nd tier test, in order to verify the analytical sensibility of this test (positive results correctly identified by the method). All seven patients were confirmed as positives by 2nd tier test and the corresponding values were in the range of 0.14–0.32 µmol/L for dGuo and 0.89–3.32 µmol/L for dIno (Table 3). No correlation was found between metabolites levels and genotype although this study was based on a limited number of patients.

The present test is of great importance not only to make diagnosis of PNP-SCID but also to achieve therapy monitoring in patients undergoing bone marrow transplantation or enzyme replacement therapy. For this purpose, DBS but also matrices as urine and plasma can be used to evaluate purine concentrations in suspected or confirmed PNP-SCID patients.

Table 3: 2′-Deoxyguanosine (dGuo), 2′-deoxyinosine (dIno) levels, genetic analysis in 7 PNP-SCID patients.

Patient	Age at DBS collection	dGuo, µmol/L	dIno, µmol/L	Genotype
P1	11 months	0.19	3.32	c.287delT; V96fs/c.548delA; E183fs
P2	3 years and 9 months	0.14	0.89	IVS3-18 G>A/ IVS3-18 G>A
P3	1 year and 3 months	0.29	0.92	P198L/P198L
P4	13 years	0.17	1.50	A117T/A117T
P5	3 years and 6 months	0.28	2.81	R58X/E89X
P6	At birth	0.32	2.56	D128G/D128G
P7	3 years and 5 months	0.46	1.43	c.387_389 delCAT/c.387_389 delCAT

Clinical findings and genetic data have been previously reported in la Marca et al. [14].

Conclusions

A critical limitation of the method for some validation parameters is represented by QC sample preparation. However, the aim of this work was the development of a method with a high specificity, thanks to the chromatographic separation before mass spectrometry detection, able to reduce false positive rate in NBS test. The method was found to be accurate, precise and reproducible and could be used in routine determination of PNP-SCID metabolites.

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