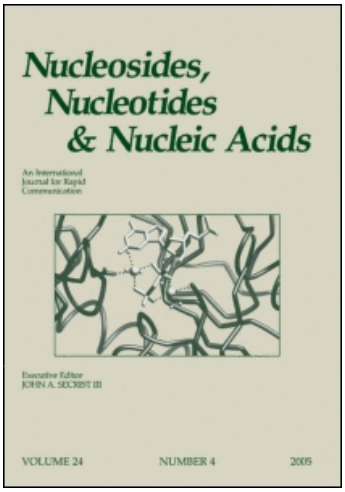

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Identification of Purine Nucleoside Phosphorylase Deficiency in Dried Blood Spots by a Non-Radiochemical Assay Using Reversed-Phase High-Performance Liquid Chromatography

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Online publication date: 11 June 2010

To cite this Article van Kuilenburg, A. B. P. , Zoetekouw, L. , Meijer, J. and Kuijpers, T. W.(2010) 'Identification of Purine Nucleoside Phosphorylase Deficiency in Dried Blood Spots by a Non-Radiochemical Assay Using Reversed-Phase High-Performance Liquid Chromatography', Nucleosides, Nucleotides and Nucleic Acids, 29: 4, 466 — 470
To link to this Article: DOI: 10.1080/15257771003741455
URL: http://dx.doi.org/10.1080/15257771003741455

Nucleosides, Nucleotides and Nucleic Acids, 29:466–470, 2010

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257771003741455

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IDENTIFICATION OF PURINE NUCLEOSIDE PHOSPHORYLASE DEFICIENCY IN DRIED BLOOD SPOTS BY A NON-RADIOCHEMICAL ASSAY USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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□ *Purine nucleoside phosphorylase (PNP) deficiency results in severe T cell dysfunction and hypouricemia. An assay to measure PNP activity in dried blood spots was developed using reversed-phase HPLC. The assay was linear with reaction times between 5 and 12.5 minutes, and protein concentrations ranging from 0.4 to 1.8 mg/ml. The intra-assay CV and the inter-assay CV for the complete assay was* $< 3.6\%$. *The PNP activity in a control blood spot, stored at 4° C, remained stable for at least one year. In a patient suffering from a PNP deficiency, the residual PNP activity was only 0.3% compared to that observed in controls (1431* $\pm$ *238 nmol/mg/h,* $n = 114$*). The PNP activity (483* $\pm$ *35 nmol/mg/h,* $n = 3$*) in heterozygotes for the c.614A > C mutation (p.E205A) in the PNP gene was 34% compared to controls. Thus, the analysis of the PNP activity in blood spots can readily detect patients with a PNP deficiency.*

Keywords Purine nucleoside phosphorylase; blood spots; SCID; *NP* gene

INTRODUCTION

Purine nucleoside phosphorylase (PNP) is an enzyme of the purine salvage pathway and catalyses the conversion of (deoxy)inosine and (deoxy)guanosine to hypoxanthine and guanine, respectively. Patients with PNP deficiency suffer from recurrent infections, neurological impairment and lymphoid malignancy, resulting in early fatality unless rescued by hematopoietic stem cell transplantation.[1,2] Patients with PNP deficiency are diagnosed by severe lymphopenia, hypouricemia, and extremely low PNP activity in red blood cell lysates.[2–4] Since the collection of blood, especially from neonates,

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might be difficult and the shipment of frozen erythrocytes expensive, the use of blood spots for analysis of PNP activity offer the advantage of easy collection, transport and storage. In this article, we developed a fast and sensitive assay of the PNP activity using dried blood spots and reversed-phase HPLC. In addition, we have established reference values for the PNP activity in the Dutch population.

MATERIALS AND METHODS

The blood spot (containing ± 10 μl EDTA blood) was soaked in 500 μl 50 mM potassium phosphate (pH 7.4) for 90 minutes and the eluate was used for analysis of PNP activity. The reaction mixture contained an aliquot of cell sample (40–180 μg), 50 mM potassium phosphate (pH 7.4) and 30 mM inosine in a total volume of 100 μl. The reaction was started by the addition of the sample. After 10 minutes incubation at 25°C, the reaction catalysed by PNP was terminated by the addition of 4 μl of ice-cold 8 M $HCLO_4$ and kept on ice for 10 minutes. After centrifugation, the resulting supernatant was saved for analysis by reversed-phase HPLC. Protein concentration in the supernatant was determined by the copper-reduction method using bicinchoninic acid, essentially as described by Smith et al.[5]

The supernatant (100 μl) was injected into the HPLC system and separation of inosine from hypoxanthine was performed using a gradient from buffer A [50 mM KH_2PO_4 (pH 6.5)] to buffer B [50 mM KH_2PO_4 (pH 6.5) and 20% (v/v) methanol] at a flow rate of 0.6 ml/min by HPLC on a reversed-phase column (Phenomenex, Torrance, CA, USA; C18 Gemini, 150 × 4.6 mm, 3 μm particle size) and a guard column with online UV detection at 260 nm. Quantification of the amounts of hypoxanthine was performed by comparison with an external standard.

DNA was isolated from EDTA-blood using the NucleoSpin Tissue kit (Macherey-Nagel, GmbH & Co. KG, Düren, Germany). PCR amplification of all 6 coding exons and flanking intronic regions of the PNP gene (*NP*) was carried out using intronic primer sets. Sequence analysis of genomic fragments amplified by PCR was carried out on an Applied Biosystems (Carlsbad, CA, USA) model 3730 automated DNA sequencer using the dye-terminator method.

RESULTS AND DISCUSSION

In this study, we developed an accurate assay for PNP using blood spots followed by separation of inosine and hypoxanthine by reversed-phase HPLC. Figure 1 shows that a complete baseline separation was obtained within 30 minutes for hypoxanthine and inosine and that the amount of hypoxanthine produced by PNP from a blood spot was readily detectable. The detection limit of hypoxanthine in the HPLC system, defined as three

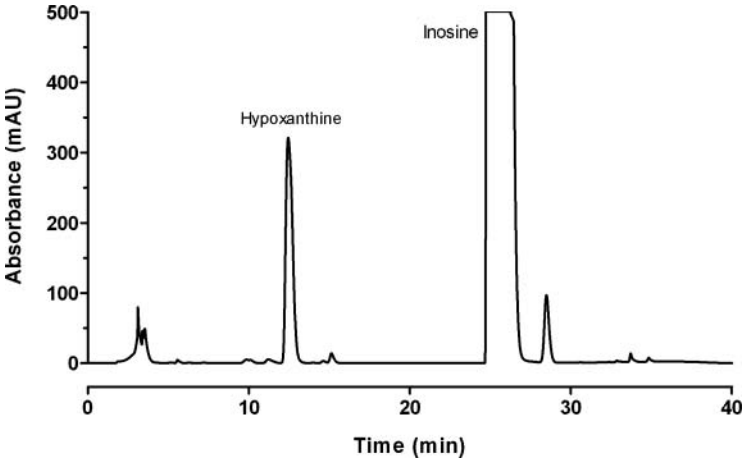

FIGURE 1 HPLC elution profile. The elution profile was obtained of a reaction mixture after incubation of a control sample for 10 minutes at 25°C.

times the value of the baseline noise, was approximately 0.5 pmol. The high activity of PNP prompted us to perform the assay at 25°C and a substrate concentration of 30 mM. This is in contrast to Jacomelli and coworkers who measured the PNP activity in bloodspots at 37°C and a substrate concentration of 1.5 mM.[6] Unfortunately, it was not reported whether their assay conditions ensured linearity of the reaction with protein and incubation time.

Figure 2 shows that, under our assay conditions, the amount of hypoxanthine produced by PNP from a blood spot increased linearly with protein concentrations ranging from 0.4 to 1.8 mg/ml and reaction times between

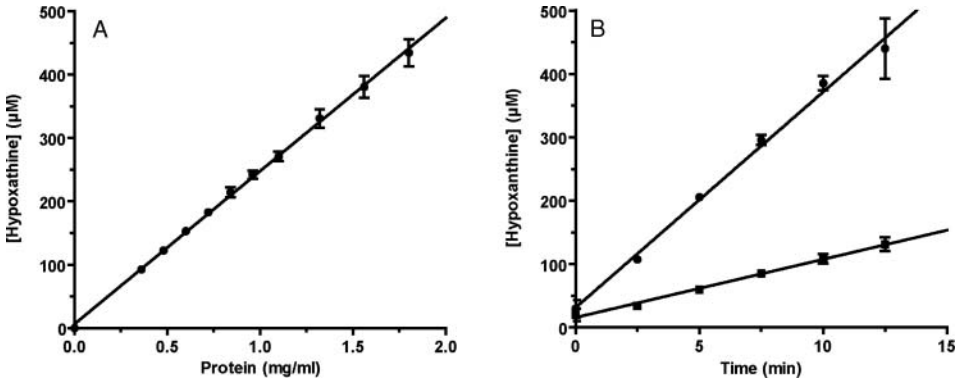

FIGURE 2 Protein dependence and time dependence of the PNP reaction. A) The amount of product (hypoxanthine) at various protein concentrations in the assay. The reaction was allowed to proceed for 10 minutes at 25°C. Each data point represents the mean of three experiments ± SD. B) The amount of product produced by PNP at various time points. The PNP activity was measured at a protein concentration of 0.4 mg/ml (■) and 1.4 mg/ml (●).

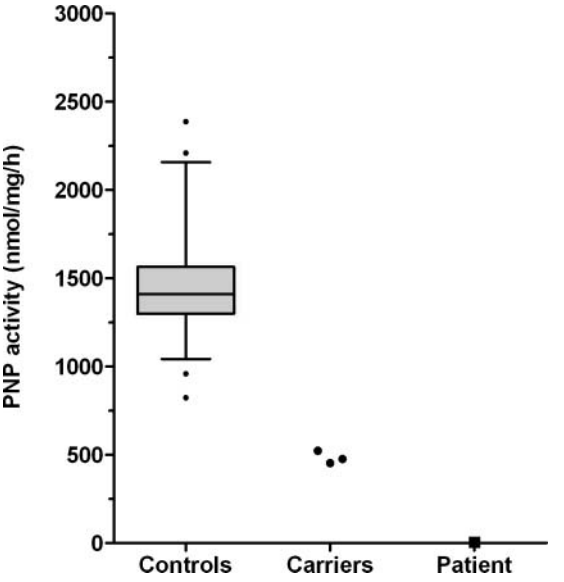

FIGURE 3 PNP activity in controls, carriers, and a PNP patient. The PNP activity in controls (n = 114) is depicted as a box plot. The whiskers on the bottom extend from the 2.5th percentile and top 97.5th percentile. The circles represent outliers. The carriers were heterozygous for the c.614A > C mutation (p. E205A) in the PNP gene. The patient was homozygous for the c.614A > C mutation.

5 and 12.5 minutes. The intra-assay CV and the inter-assay CV for the complete assay, HPLC detection and protein determination, were 3.3% ($n = 10$) and 3.6% ($n = 10$), respectively. The PNP activity (1478 ± 63 nmol/mg/h; CV 4.3%) in a control blood spot, stored at 4°C, remained stable for at least one year.

In a patient suffering from a PNP deficiency due to a novel c.614A>C mutation (E205A) in the PNP gene (*NP*), the residual PNP activity (4.5 nmol/mg/h) was only 0.3% compared to that observed in controls (1431 ± 238 nmol/mg/h, n = 114; Figure 3). The PNP activity (483 ± 35 nmol/mg/h, n = 3) in heterozygotes for the c.614A > C mutation was 34% compared to controls. Thus, the analysis of the PNP activity in blood spots can readily detect patients with a PNP deficiency.

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