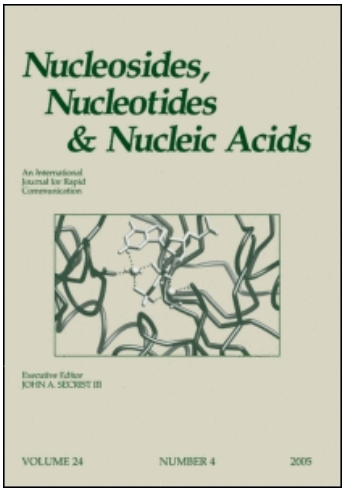

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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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) 'Determination of Adenosine Deaminase Activity in Dried Blood Spots by a Nonradiochemical Assay Using Reversed-Phase High-Performance Liquid Chromatography', Nucleosides, Nucleotides and Nucleic Acids, 29: 4, 461 — 465
To link to this Article: DOI: 10.1080/15257771003741406
URL: http://dx.doi.org/10.1080/15257771003741406

Nucleosides, Nucleotides and Nucleic Acids, 29:461–465, 2010

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257771003741406

DETERMINATION OF ADENOSINE DEAMINASE ACTIVITY IN DRIED BLOOD SPOTS BY A NONRADIOCHEMICAL ASSAY USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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□ *Adenosine deaminase (ADA) deficiency is a rare metabolic disease causing severe combined immunodeficiency (SCID). An assay to determine ADA activity in dried blood spots was developed using reversed-phase HPLC. The assay was linear with reaction times up to at least 4 hours, and protein concentrations up to at least 2.2 mg/ml. The intra-assay CV and the inter-assay CV for the complete assay was 3.5 and 8.4%, respectively. The ADA activity in a control blood spot, stored at 4° C, remained stable for at least one year. Only a slightly decreased ADA activity (35 ± 13 nmol/mg/h, n = 4) was observed in heterozygotes for a c.704G > A mutation in the ADA gene when compared to that observed in controls (41 ± 13 nmol/mg/h, n = 108). In addition, increased ADA activity as found in a rare form of congenital anemia can be assessed, as observed in a bloodspot from a patient diagnosed with Diamond Blackfan anemia (ADA activity 150 nmol/mg/h).*

Keywords Adenosine deaminase; blood spots; SCID; DBA; *ADA*

INTRODUCTION

Adenosine deaminase (ADA) is an enzyme of the purine salvage pathway that catalyses the conversion of (deoxy)adenosine to (deoxy)inosine. Patients with ADA deficiency suffer from severe combined immunodeficiency (SCID) in which all lymphoid lineages are affected.[1,2] In addition to the profound lymphopenia, the toxic levels of deoxyadenosine and dATP can give rise to hepatic, skeletal, neurological and behavioral abnormalities. ADA deficiency is diagnosed by low ADA activity in red blood cell lysates. In contrast, patients with Diamond Blackfan anemia (DBA), which is a rare,

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genetically and clinically heterogeneous, inherited red cell aplasia, present with strongly elevated ADA activity in their erythrocytes.[3] In this article, we developed a fast and sensitive assay to measure ADA activity in dried blood spots using reversed-phase HPLC.

MATERIALS AND METHODS

The blood spot (±30 μl) was soaked in 500 μl 50 mM potassium phosphate (pH 7.4) for 90 minutes and the eluate was used for analysis of ADA activity. The reaction mixture contained an aliquot of cell sample (12–220 μg), 50 mM potassium phosphate (pH 7.4) and 500 μM adenosine in a total volume of 100 μl. The reaction was started by the addition of the sample. After 2 hours incubation at 37°C, the reaction catalyzed by ADA 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, as described by Smith et al.[4]

The supernatant (100 μl) was injected into the HPLC system and separation of adenosine from inosine and 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 inosine and hypoxanthine was performed by comparison with an external standard. The amount of both inosine and hypoxanthine produced were added together to determine the amount of ADA activity.

DNA was isolated from EDTA-blood using the NucleoSpin Tissue kit (Macherey-Nagel, GmbH & Co. KG, Düren, Germany). PCR amplification of all 12 coding exons and flanking intronic regions of the *ADA* gene was carried out using intronic primer sets. Sequence analysis of genomic fragments amplified by PCR was carried out on an Applied Biosystems model 3730 automated DNA sequencer using the dye-terminator method.

RESULTS AND DISCUSSION

In this study, we developed an accurate assay to assess ADA activity in blood spots. Figure 1 shows that a complete baseline separation was obtained within 40 minutes for adenosine, inosine and hypoxanthine and that the amount of inosine and hypoxanthine produced by ADA and purine nucleoside phosphorylase, respectively, from a blood spot was readily detectable. The detection limit of inosine and hypoxanthine in the HPLC

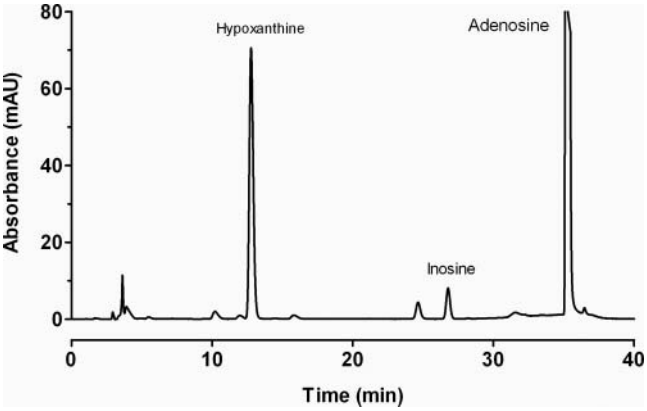

FIGURE 1 HPLC elution profile. The elution profile was obtained of a reaction mixture after incubation of a control sample for 2 hours at 37°C.

system, defined as three times the value of the baseline noise, was approximately 3.1 pmol and 0.5 pmol, respectively. Figure 2 shows that the ADA activity from a blood spot increased linearly with protein concentrations up to 2.2 mg/ml and reaction times up to at least 4 hours. The steady-state kinetics of ADA with adenosine showed an apparent K_m value of 32 μM (Figure 3). The intra-assay CV and the inter-assay CV for the complete assay, HPLC detection and protein determination, were 3.5% ($n = 10$) and 8.4% ($n = 10$), respectively. The ADA activity (35.7 ± 3.3 nmol/mg/h, CV = 9.3%) in a control blood spot, stored at 4°C, remained stable for at least one year.

Homozygosity for the c.704G > A mutation (p.R235Q) in the *ADA* gene resulted in a near complete ADA deficiency.[2] In carriers for the mutation,

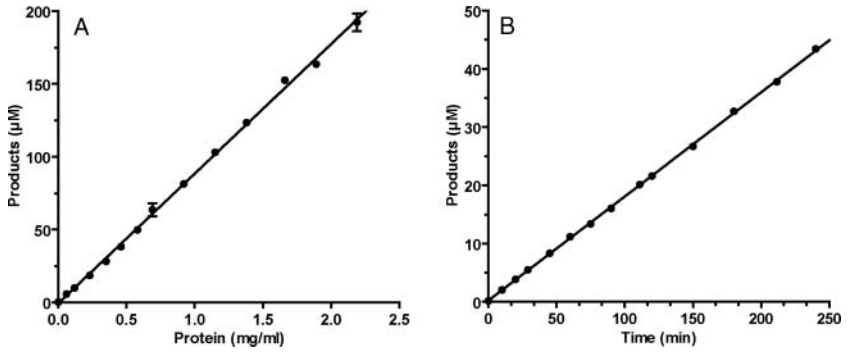

FIGURE 2 Protein dependence and time dependence of the ADA reaction. A) The amount of product (inosine + hypoxanthine) at various protein concentrations in the assay. The reaction was allowed to proceed for 2 hours at 37°C. Each data point represents the mean of three experiments ± SD. B) The amount of product (inosine + hypoxanthine) produced by ADA at various time points. The ADA activity was measured at a protein concentration of 0.25 mg/ml.

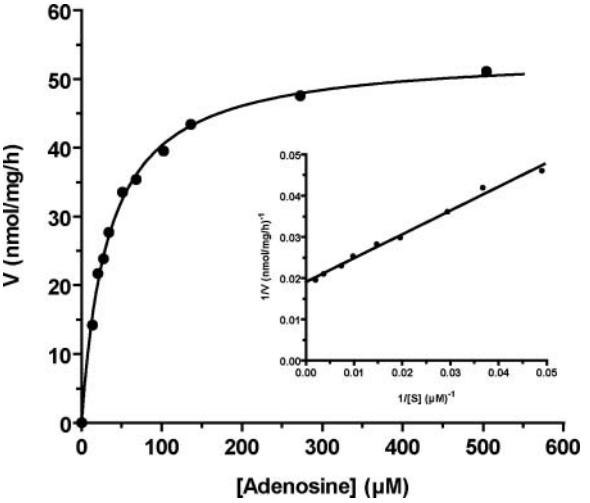

FIGURE 3 Steady state kinetics of ADA. The insert shows the double reciprocal plot of the reaction velocity versus the concentration of adenosine.

only a slightly reduced activity was observed when compared to control values (41 ± 13 nmol/mg/h, n = 108; Figure 4). Strongly *increased* ADA activity (150 nmol/mg/h) was observed in a bloodspot from a neonate (11 months old) diagnosed with DBA, as reported previously for erythrocyte lysates.[3] Thus, our results indicate that the analysis of the ADA activity in blood spots can be used to accurately determine enzymatic activity for diagnostic purposes.

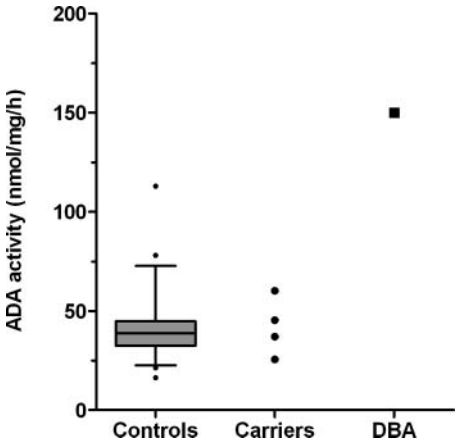

FIGURE 4 ADA activity in controls, carriers and a DBA patient. The ADA activity in controls is depicted as a box plot. The whiskers on the bottom extend from the 2.5th percentile and top 97.5th percentile. The circles represent outliers. The carriers were heterozygous for the c.704G > A mutation (p. R235Q) in the ADA gene. DBA, Diamond Blackfan Anemia.

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