



# Development of dried blood spot quality control materials for adenosine deaminase severe combined immunodeficiency and an LC-MS/MS method for their characterization

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## ABSTRACT

Adenosine deaminase severe combined immunodeficiency (ADA-SCID) is an autosomal recessive disorder in which a lack of ADA enzyme prevents the maturation of T- and B-cells; early intervention is crucial for restoring immune function in affected neonates. ADA is responsible for purine metabolism and—in its absence—adenosine, deoxyadenosine, and S-adenosylhomocysteine build up and can be detected in the blood. Preparing dried blood spot (DBS) quality control (QC) materials for these analytes is challenging because enrichments are quickly metabolized by the endogenous ADA in normal donor blood. Adding an inhibitor, erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), has been previously reported to minimize enzyme activity, although this adds additional cost and complexity. We describe an alternative method using unnatural L-enantiomer nucleosides (L-adenosine and L-2'-deoxyadenosine) which eliminates the need for enzyme inhibition. We also present a novel method for characterization of the materials using liquid chromatography mass spectrometry to quantify the analytes of interest.

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## 1. Introduction

Adenosine deaminase severe combined immunodeficiency (ADA-SCID, OMIM ID: 102700) is a sub-type of SCID, a group of congenital diseases that weaken the immune system. Infants with early-onset ADA-SCID appear healthy at birth, but die in infancy of recurring infections unless adequate therapy is provided [1–4]. Unfortunately, some infants with ADA-SCID are not detected in

*Abbreviations:* 2'-dIno, deoxyinosine; AA, amino acid; AC, acylcarnitine; ADA-SCID, adenosine deaminase severe combined immunodeficiency; CSS, charcoal stripped serum; D-2'-dAdo, deoxyadenosine (D enantiomer); D-Ado, adenosine (D enantiomer); DBS, dried blood spot; EHNA, erythro-9-(2-hydroxy-3-nonyl) adenine; ESI, electrospray ionization; FIA, flow injection analysis; Ino, inosine; L-2'-dAdo, deoxyadenosine (L enantiomer); L-Ado, adenosine (L enantiomer); LC-MS/MS, liquid chromatography tandem mass spectrometry; NBS, newborn screening; RBC, red blood cell; RT-qPCR, real-time quantitative polymerase chain reaction; QC, quality control; SRM, selected reaction monitoring; SUAC, succinylacetone; TRECS, t-cell receptor excision circles.

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the pre-infection period and the diagnosis occurs only after an infection has escalated. Damage caused by severe infections may be irreversible or fatal. Therefore, early detection of the disease through newborn screening is vital to patients and their families.

ADA-SCID is an autosomal recessive disorder with an incidence of 1:375,000 to 660,000 live births and accounts for 15% to 20% of all SCID cases [4]. The mutation is in the gene for isoform ADA1, a component of the purine salvage pathway where it catalyzes the deamination of naturally occurring (i.e., D-β configuration) adenosine (D-Ado) and 2'-deoxyadenosine (D-2'-dAdo) into inosine (D-Ino) and 2'-deoxyinosine (D-2'-dIno), respectively. ADA1 activity is associated with lymphocytes and macrophages [5], while the isoform ADA2 is secreted into plasma by differentiated monocytes [5,6]. Loss of ADA2 activity does not result in ADA-SCID [7]. In the early-onset form, which accounts for approximately 85% to 90% of ADA-SCID [4], the absence of ADA1 activity leads to an accumulation of D-Ado and D-2'-dAdo in immune cells. The latter nucleoside inhibits DNA synthesis in maturing lymphocytes, resulting in defects of the immune system and permanent damage to other organs such as the brain and liver [4,8,9]. In these cases,

ADA-SCID is usually fatal if not treated within the first few months of the individual's life.

The most common method for detecting ADA-SCID utilizes real-time quantitative polymerase chain reaction (RT-qPCR) to analyze T-cell receptor excision circles (TRECs) in dried blood spots (DBS) collected from newborns [1]. These TRECs are an indication of immune cell maturation and, while this method can identify early onset ADA-SCID, it cannot identify delayed or late-onset ADA-SCID [10]. Previous work using flow injection analysis electrospray ionization tandem mass spectrometry (FIA-ESI-MS/MS) [11] demonstrated that Ado and 2'-dAdo levels were elevated in both early- and late-onset forms of ADA-SCID in DBS from newborns.

Preparing quality control (QC) materials for this disease is challenging, as the healthy donor blood used to make the QC blood matrix contains active ADA enzyme. The ADA inhibitor erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) can sufficiently inhibit the enzyme in whole blood, allowing for the preparation of DBS enriched with D-Ado and D-2'-dAdo [12]. However, EHNA is most effective at moderately high concentrations (>80  $\mu\text{M/L}$ ) and including it in every QC sample and calibrator will increase the cost of DBS production along with the demands of sample preparation. In terms of nucleoside quantitation, various analytical methods have been used to separate and analyze nucleosides, including capillary electrophoresis [13], reversed-phase chromatography [14,15], and hydrophilic interaction chromatography (HILIC) [16–18] coupled with ultraviolet or mass spectrometry detectors. Furthermore, the non-derivatized NeoBase™ 2 (PerkinElmer, MA, USA) assay is an FIA-ESI-MS/MS method that simultaneously detects Ado and 2'-dAdo for the screening of ADA-SCID in newborns.

In this study, we investigate an alternative method for preparing stable QC material for ADA-SCID screening that decreases costs, complexity, and time handling enriched blood, using unnatural L-counterparts L-adenosine (L-Ado) and L-2'-deoxyadenosine (L-2'-dAdo). These L-nucleosides are enantiomers of the naturally occurring D-nucleosides and have very similar physical and chemical properties (Fig. 1). Using any achiral analytical method, such as FIA-ESI-MS/MS or LC-MS/MS, an L-nucleoside will be indistinguishable from its D-counterpart. In addition, these unnatural molecules typically display an increased resistance to enzymatic degradation. Human ADA cannot process L-Ado [19], indicating these L-nucleosides might be valuable tools in the production of matrix-matched QC and calibrators for ADA-SCID screening.

Furthermore, a high-throughput fit-for-purpose HILIC LC-MS/MS method was developed that provides baseline resolution and the ability to quantify Ado, 2'-dAdo, and their respective ADA enzyme products, Ino and 2'-dIno, in DBS, showing that D-Ado is indeed metabolized to its product, D-Ino, when not recovered. Finally, a previously developed non-derivatized screening assay for nine amino acids (AA), 18 acylcarnitines (AC), and succinylacetone (SUAC) [20] was modified to include the analysis of Ado and 2'-dAdo, adding ADA-SCID to the number of disorders already being screened for in the non-derivatized newborn screening assay.

## 2. Materials and methods

### 2.1. Materials

Solvents (water, methanol, acetonitrile, and formic acid) were LC-MS grade from Fisher Scientific. D-Ado, D-2'-dAdo, and erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) were purchased from Sigma Aldrich (St. Louis, MO). D- $\alpha$ -Adenosine ( $\alpha$ -Ado) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). L-Ado and L-2'-dAdo were purchased from Carbosynth (San Diego, CA).

$^{13}\text{C}_5$ -Ribose-adenosine and  $^{13}\text{C}_5$ -Ribose-2'-deoxyadenosine were purchased from Cambridge Isotope Laboratories (Andover, MA).

### 2.2. Blood matrix

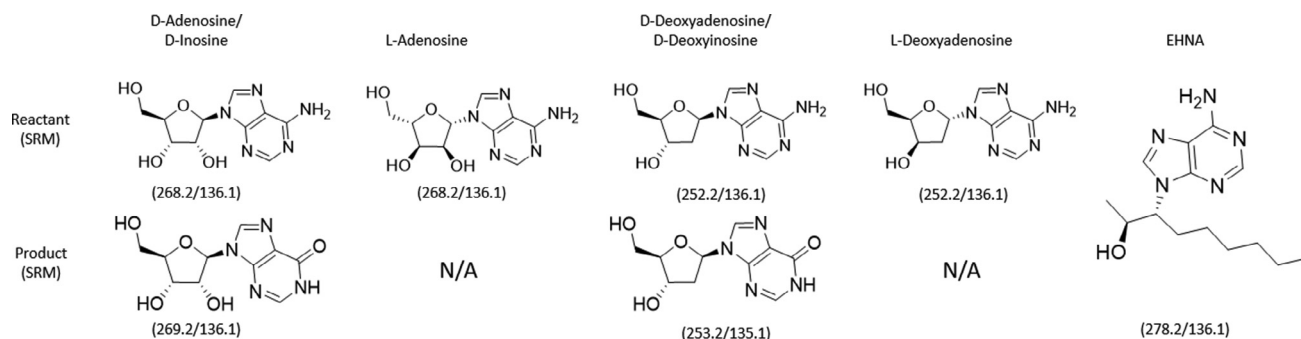
Institutional Review Board (IRB)/Ethics Committee ruled that approval was not required for this study. Blood production was performed as previously described [21,22] and is outlined below. For blood matrix preparation, packed human red blood cells (RBC) (type O + ) were purchased from Tennessee Blood Services. Human charcoal-stripped serum (CSS) was purchased from SeraCare LifeSciences (Milford, MA). Equal amounts of red blood cells and saline (Thermo Scientific, isotonic, 0.9% w/v) were mixed by inverting 5–10 times. The RBCs were then centrifuged for 10 min at 3200 rpm at 10 °C. The saline layer was removed using vacuum aspiration. The process was repeated two additional times. During the third wash, the RBCs and saline were centrifuged for 15 min at 4000 rpm at 10 °C. All saline was aspirated off the RBCs. The hematocrit was adjusted to 50%  $\pm$ 1% (Drummond Hemato-Clad Mylar-Wrapped 75 mm Hematocrit Tubes, IEC Microhematocrit Centrifuge, Fisher Scientific) using CSS. The blood was transferred to polypropylene Nalgene bottles and stored at  $-20$  °C for 2 weeks to obtain freeze-thawed blood matrix (lysed erythrocytes). It was determined that the present study does not fall under human subject research as it used deidentified commercial blood and serum products.

### 2.3. Blood enrichment and DBS preparation

The blood matrix was divided into thirteen pools. EHNA was added to 9 pools in increasing concentrations (0–160  $\mu\text{M}$ ) and stirred for 3 h at room temperature, after which D-Ado and D-2'-dAdo were added at 10  $\mu\text{M}$  while stirring. After 10 min, each blood pool was spotted in 75  $\mu\text{L}$  aliquots onto grade 903 filter paper (Eastern Business Forms, Greenville, SC) and dried overnight at room temperature. To the final 4 pools, L-nucleosides were added directly to blood (0–10  $\mu\text{M}$ ), stirred for 10 min, spotted onto grade 903 filter paper, and dried overnight at room temperature. The spotted filter paper was placed in airtight plastic storage bags with desiccant and a humidity indicating card, then stored at  $-20$  °C.

### 2.4. Sample preparation

Single 1/8" (3.1 mm) disks punched from QC DBS were placed in wells of a polypropylene 96-well plate (VWR, Vineland, NJ) and 100  $\mu\text{L}$  of 80:20 acetonitrile/water with 0.05% formic acid and 0.015% hydrazine, containing stable isotope-labeled internal standard, was added to each well. The concentrations and SRM transitions of AA, AC, SUAC, and nucleotide internal standards are shown in Supplemental Table 1. The 96-well plate was heat sealed with aluminum foil and incubated at 45 °C for 45 min with shaking (MaxQ 4450 Shaker, Thermo Scientific). The extract was then transferred to a new 96-well plate, followed by drying with nitrogen (Peak Scientific, Inchinnan, Scotland) at 50 °C for 20 min. In the methods analyzing SUAC, 50  $\mu\text{L}$  of methanol was added to each well and dried with nitrogen at 50 °C for 15 min to remove hydrazine present in the internal standard solution. Finally, 100  $\mu\text{L}$  of mobile phase (50:50 acetonitrile/water with 0.02% formic acid) was added to each well and the plate was heat-sealed with aluminum foil and shaken for 3 min prior to FIA-ESI-MS/MS analysis. Samples were extracted with either acetonitrile/water or methanol/water at 100, 90, 80, or 70% organic solvent containing  $^{13}\text{C}_5$ -ribose-adenosine and  $^{13}\text{C}_5$ -ribose-deoxyadenosine as the only internal standards, then reconstituted in a mobile phase of 97:3 acetonitrile/water. All other steps remained the same.



**Fig. 1.** D- and L-enantiomers of adenosine (Ado) and deoxyadenosine (dAdo) with respective ADA1 products and enzyme inhibitor, erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA).

**Table 1**  
Parameters for the Waters Xevo TQD, I Class Acquity UPLC system using FIA-ESI-MS/MS specific to ADA-SCID.

Source-dependent parameters				
CAD gas	Capillary voltage	Source temperature	Desolvation temperature	Desolvation rate
3(10 <sup>-3</sup> ) Torr	3000 V	150°C	400°C	650 L/hr
Compound-dependent parameters				
Compound	Collision energy	Cone voltage	Dwell time	
Adenosine	19 V	36 V	0.05 s	
Deoxyadenosine	20 V	31 V	0.05 s	
Scan-dependent parameters				
Adenosine SRM	<sup>13</sup> C <sub>5</sub> -adenosine SRM	Deoxyadenosine SRM	<sup>13</sup> C <sub>5</sub> -deoxyadenosine SRM	
268.2 → 136.1	273.2 → 136.1	252.2 → 136.1	257.2 → 136.1	

**2.5 Flow injection analysis Electrospray tandem mass spectrometry (FIA-ESI-MS/MS).** During FIA, an in-line stainless steel filter (0.094 × 0.065 in, 2 μm) was used with a Waters I Class Acquity UPLC system (Waters Corporation, Milford Massachusetts) with a mobile phase composed of 50:50 acetonitrile/water with 0.02% formic acid. A 10 μL injection of the sample was analyzed. Total run time was 1.0 min with elution of the analytes at 0.50 min and maximum typical back pressure of approximately 300 psi. The initial flow rate was 100 μL/min; at 0.25 min flow was decreased to 20 μL/min; at 0.75 min flow was increased to 600 μL/min; and between 0.85 and 1.00 min flow was decreased to 100 μL/min. This inlet program is identical to one used during a typical AA, AC, and SUAC FIA-ESI-MS/MS assay [23].

A Waters Xevo TQD triple quadrupole mass spectrometer in electrospray positive mode was used with the source-dependent parameters and compound-dependent parameters shown in Table 1. Four positive-mode selected reaction monitoring (SRM) pairs for unlabeled and labeled Ado and 2'-dAdo were added to a method containing 54 SRM pairs for AA, AC, SUAC, and their internal standards. The SRM pairs for all quantified unlabeled analytes are shown in Supplemental Table 1. Fragment ions, collision energy, and cone voltage for Ado and 2'-dAdo were determined by infusion of the individual compounds (3 μM in mobile phase). Amino acid SRM pairs—except for citrulline, ornithine, and arginine—corresponded to a neutral loss of 46 Da and were optimized by infusion as above (2.5 μM in mobile phase). Except for free carnitine (C0), acylcarnitine SRM pairs corresponded to a product ion of *m/z* 85 and optimization was performed by infusion as above (0.08 or 0.16 μM in mobile phase). The concentration of each analyte was calculated by multiplying the ratio of analyte peak area and corresponding internal standard peak area by the concentration of internal standard in extraction solvent (Table S2) and by the dilution of 3.1 μL of blood from the 3.1 mm DBS disk into 100 μL of extraction solvent.

## 2.5. LC-MS/MS Method development

A < 5 min LC-MS/MS method for the separation of Ado and 2'-dAdo from their metabolites, Ino and 2'-dIno, was developed on a Waters I Class Acquity UPLC system. Sample detection and quantification was performed on a Waters Xevo TQD triple quadrupole mass spectrometer in positive electrospray mode as defined above. 10 μL of sample prepared as described in section 2.4, or sample mixtures prepared from liquid calibrators, were injected onto a Supelcosil™ LC-NH<sub>2</sub> 2.1 × 50 mm, 3 μm HILIC column (Sigma Aldrich, St. Louis, MO) for separation. The samples were eluted with a flow rate of 0.4 mL/min before a quick column wash at 1.2 mL/min. See Table 2 for mobile phase and gradient conditions.

## 2.6. Linearity, Precision, Recovery

The linearity, precision, and analyte recovery for the FIA-ESI-MS/MS and LC-MS/MS methods were investigated in DBS enriched with the L-nucleosides. Using labeled internal standards <sup>13</sup>C<sub>5</sub>-Ado and <sup>13</sup>C<sub>5</sub>-dAdo at a constant concentration of 3 μM, sample linearity of L-Ado and L-2'-dAdo was measured in DBS at concentrations of 0, 1, 5, and 10 μM in 70:30 MeOH/H<sub>2</sub>O. Six replicates per sample were measured over a single day and the mean measured concentration was plotted against the expected value (Fig. 2). Samples were linear over the range tested. Sample recovery was defined as the calculated concentration extracted from each set of dried blood spots divided by the expected value of the analyte (amount found in lysed base pool plus the amount added). These values correlated well with the prepared standard curve.

## 2.7. Statistical methods

Percent recovery was calculated as (measured concentration/expected concentration) × 100. Precision was measured as the

**Table 2**

Mobile phase and gradient conditions in HILIC LC-MS/MS method specific to ADA-SCID. Mobile phase (A): 97:3 acetonitrile/water, with 1% formic acid and mobile phase (B): 100% water with 1% formic acid.

Time (min)	Flow rate (mL/min)	% A	% B
0.00	0.400	100	0
1.00	0.400	100	0
2.50	0.400	93	7
4.00	0.400	88	12
4.10	0.400	100	0
5.00	1.20	100	0
5.20	0.400	100	0

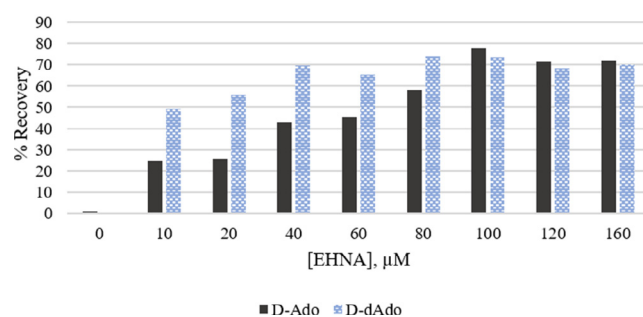
relative peak area in reference to a known concentration of internal standard. Intraday precision was calculated as the (standard deviation/measured average concentration)  $\times$  100 of 10 sample replicates analyzed over a 1-day period. Interday precision was calculated as the (standard deviation/measured average concentration)  $\times$  100 of 10 sample replicates analyzed over a 6 day period. Linearity of the analytes was reported as the measured concentration (six replicates) in DBS over 4 concentrations (0, 1, 5, 10  $\mu$ M) and plotted against 7 liquid calibrators of known concentration (0, 0.5, 1, 2, 5, 10, 20  $\mu$ M).

### 3. Results

#### 3.1. Optimization of blood matrix

Enrichment of whole blood (intact erythrocytes) and freeze-thawed blood (lysed erythrocytes) at concentrations of 1–10  $\mu$ M D-Ado and D-2'-dAdo resulted in poor (<1%) apparent recovery of both compounds from the resulting DBS (Fig. 3, 0  $\mu$ M EHNA). Based on the hypothesis that ADA1 or ADA2 activity in blood was responsible for the conversion of D-Ado and D-2'-dAdo into D-Ino and D-2'-dIno, respectively, we investigated different ways to increase the recovery of these nucleosides. Heat treating the blood was not enough to deactivate the enzyme responsible for the degradation of D-Ado and D-2'-dAdo (data not shown), so the use of ADA1 inhibitor, EHNA, was compared to enriching the blood matrix with the unnatural nucleosides, L-Ado and L-2'-dAdo. Ado is composed of adenine attached to a ribose sugar, therefore we explored the effects of the inhibitor, EHNA, on both the  $\beta$ - (naturally occurring in body) and  $\alpha$ -N<sub>9</sub>-glycosidic bond forms of the D-enantiomer. The  $\beta$ -form showed recovery < 35% when treated with the inhibitor, EHNA. The  $\alpha$ -form however, showed increased recovery, >70%. While EHNA showed robust inhibition of ADA1 in the DBS and enabled recoveries >70% for both D-Ado and

#### D-Ado and D-dAdo Recovery after EHNA Inhibition



**Fig. 3.** Percent recovery of 10  $\mu$ M enrichment D-( $\beta$ )-Ado and D-( $\beta$ )-2'-dAdo in DBS after EHNA inhibition (0–160  $\mu$ M), quantified using FIA-ESI-MS/MS. D-( $\beta$ )-Ado: D-( $\beta$ )-adenosine; D-( $\beta$ )-2'-dAdo: D-( $\beta$ )-2' deoxyadenosine.

D-2'-dAdo (Fig. 3), high concentrations of EHNA were required (>80  $\mu$ M). In comparison, enriching lysed blood with the unnatural L-nucleosides yielded consistently higher recoveries (>80%, Table 3) without the need for any further additions to the blood matrix.

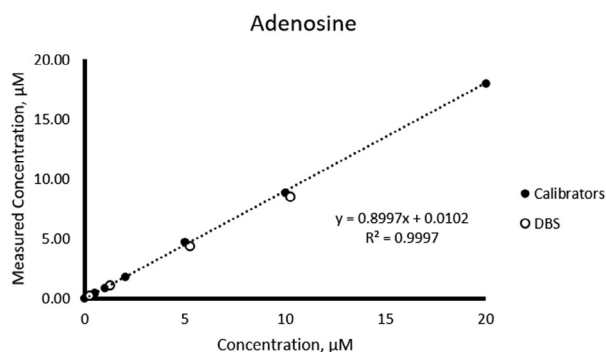
#### 3.2. Multiplexed DBS extraction

Extraction of Ado, 2'-dAdo, from DBS was multiplexed into our AA, AC, and SUAC FIA-ESI-MS/MS method, and an acceptable recovery of these analytes was obtained using the 45 min incubation at 45  $^{\circ}$ C. Since several different extraction solvents have traditionally been used to extract small molecules from DBS specimens, the extraction efficiency of Ado and 2'-dAdo was tested with a range of likely solvent choices. Eight extraction conditions were tested, from 70 to 100%, either acetonitrile or methanol in water, demonstrating that this pair of analytes can be quickly added to most day-to-day screening protocols employed by clinical laboratories (Fig. 4).

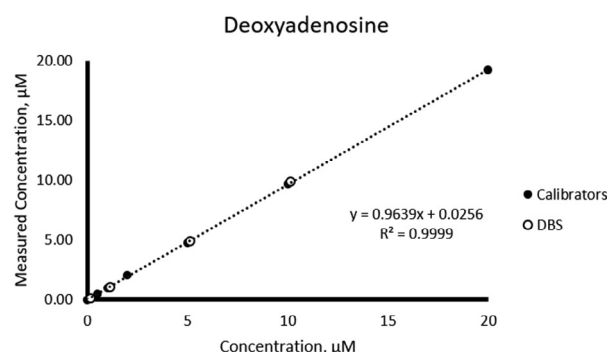
#### 3.3. FIA-ESI-MS/MS analysis of QC DBS containing AA, AC, and SUAC

Method 1 consisted of 60 SRM pairs, 4 corresponding to unlabeled and labeled Ado and 2'-dAdo, and 56 corresponding to labeled and unlabeled AA, AC, and SUAC (Table S1). Method 2 consisted of 54 SRM pairs, corresponding to labeled and unlabeled AA, AC and SUAC analytes. Both methods were used to analyze QC DBS enriched with low and high concentrations of AA, AC, and SUAC. The results indicated the concentrations of AA, AC, and SUAC were

A.



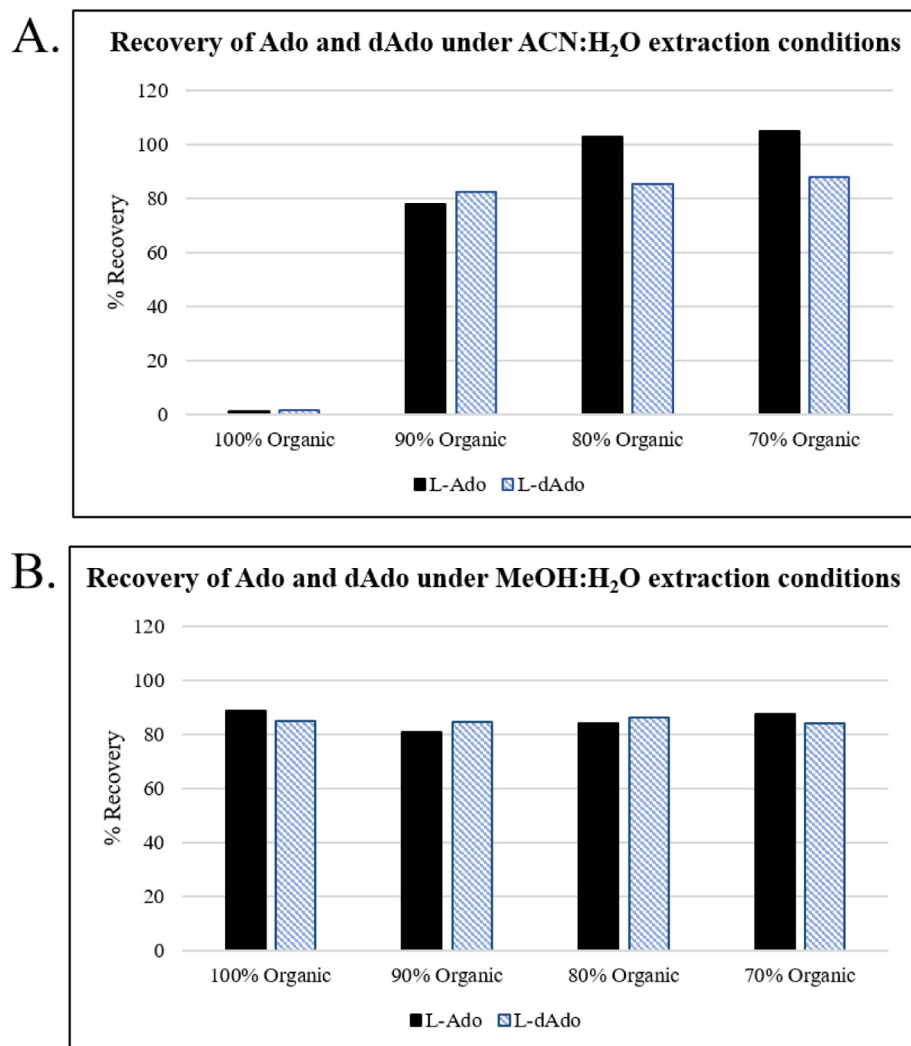
B.



**Fig. 2.** FIA-ESI-MS/MS linearity of L-Ado (A) and L-2'-dAdo (B) in DBS, measured over four concentrations (0, 1, 5, 10  $\mu$ M) and plotted against seven calibrator concentrations (0, 0.5, 1, 2, 5, 10, 20  $\mu$ M). Ado: adenosine; 2'dAdo: 2' deoxyadenosine.

**Table 3**  
Precision and recovery of FIA-ESI-MS/MS analysis. Average measured concentration, intraday precision, interday precision, and percent recovery of L-Ado and L-2'-dAdo in enriched (0, 1, 5, 10  $\mu\text{M}$ ) DBS without the addition of EHNA, quantified using FIA-ESI-MS/MS. Samples were measured in replicates of ten and collected over a six-day period.

Analyte	Added concentration ( $\mu\text{M/L}$ )	Measured concentration ( $\mu\text{M/L}$ )	Within-day CV	Between-day CV	Recovery mean (%)
L-Ado	0	0.3	23.7	35.6	NA
	1	1.1	12.3	14.9	84.0
	5	4.4	4.5	6.1	83.2
	10	8.5	6.8	7.0	83.1
L-2'-dAdo	0	0.2	36.9	45.7	NA
	1	1.1	7.2	12.4	91.4
	5	4.9	4.0	4.8	94.8
	10	9.9	5.0	6.8	97.5



**Fig. 4.** Percent recovery of L-nucleosides in DBS when extracted under various organic solvent conditions of (A) ACN: H<sub>2</sub>O and (B) MeOH: H<sub>2</sub>O. Samples of known concentrations (1, 5, 10  $\mu\text{M}$ ) were quantified using FIA-ESI-MS/MS in duplicate and the average recovery of the three samples ( $n = 6$ ) reported.

not significantly different, and all analytes overlapped at the 95% confidence interval.

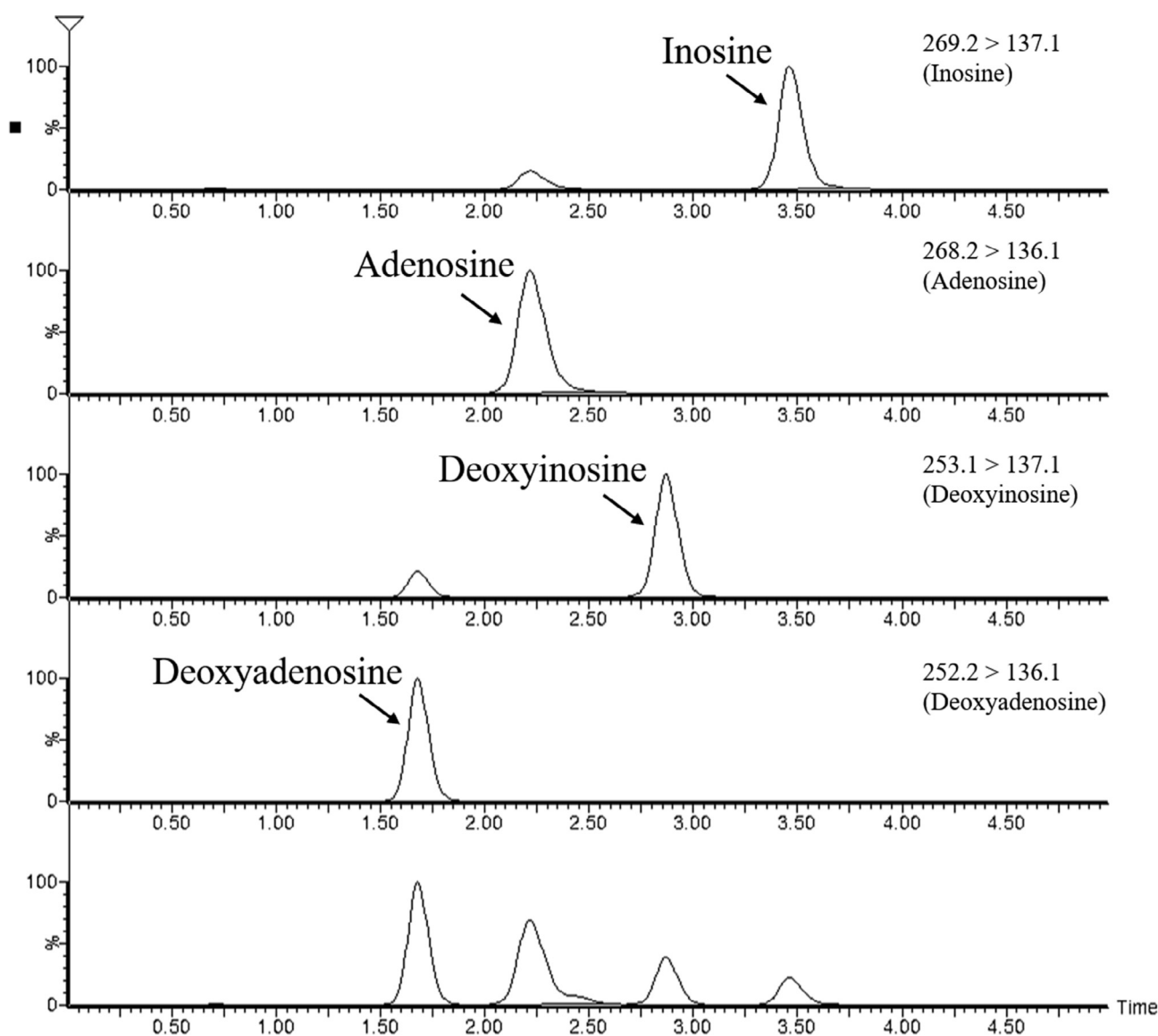
#### 3.4. FIA-ESI-MS/MS analysis of QC DBS containing L-Ado and L-2'-dAdo or EHNA with D-Ado and D-2'-dAdo

Method 1 (see section 3.3) was used to analyze QC DBS enriched with 10  $\mu\text{M}$  each of D-Ado and D-2'-dAdo with increasing concentrations of EHNA (Fig. 3). In the same fashion, this method was used to analyze QC DBS enriched with 0, 1, 5 or 10  $\mu\text{M}$  each of L-Ado with L-2'-dAdo (Table 3). While EHNA was capable of inhibit-

ing the activity of ADA in dried blood spots, recoveries of the L-nucleosides were consistently higher than their D-counterparts.

#### 3.5. LC-MS/MS analysis of QC DBS containing L-Ado and L-2'-dAdo

A short LC-MS/MS method (Table 2) was developed, capable of separating Ado and 2'-dAdo from Ino and 2'-dIno, the products of the reaction catalyzed by ADA1 (Fig. 5). This provided additional confidence in quantification through verification of the retention times of Ado and 2'-dAdo, and correctly identified any  $M + 1$  isotopic spillover that would be falsely counted as Ino/2'-dIno signal.



**Fig. 5.** LC-MS/MS chromatograms of adenosine, deoxyadenosine and their respective products, inosine and deoxyinosine (panels 1–4). All compounds can be separated (panel 5). Acetonitrile and water with 1% formic acid were used as mobile phases over a gradient elution (Table 2).

**Table 5**

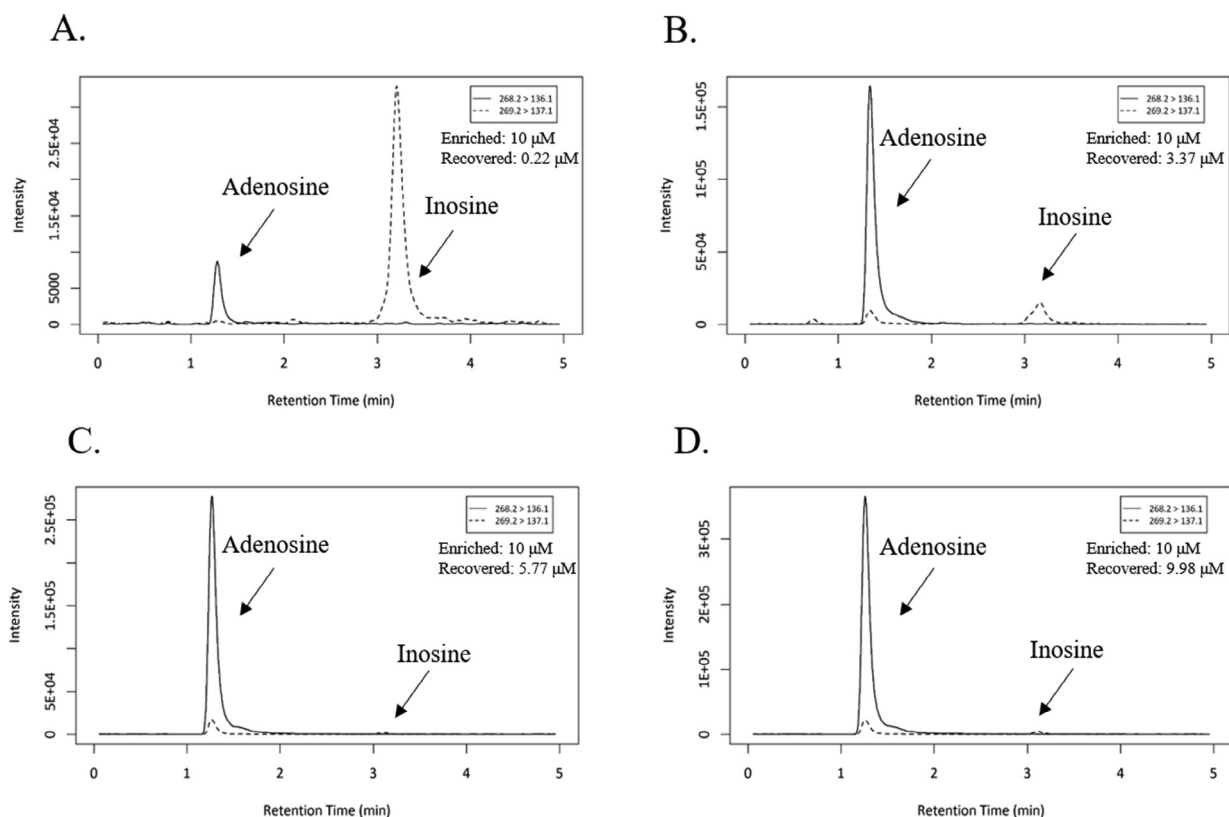
Precision and recovery of LC-MS/MS analysis. Average measured concentration, intraday precision, interday precision, and percent recovery of L-Ado and 2'-L-dAdo in enriched (0, 1, 5, 10  $\mu\text{M}$ ) DBS quantified using LC-MS/MS. Samples were measured in replicates of ten and collected over a six-day period.

Analyte	Added concentration ( $\mu\text{M}/\text{L}$ )	Measured concentration ( $\mu\text{M}/\text{L}$ )	Within-day CV	Between-day CV	Recovery mean (%)
L-Ado	0	0.3	22.4	9.0	NA
	1	1.2	11.1	4.0	93.1
	5	4.8	6.8	4.4	89.7
	10	9.5	6.7	4.9	92.1
2'-L-dAdo	0	0.0	80.0	9.0	NA
	1	0.9	6.8	4.0	88.3
	5	4.6	4.5	4.4	91.8
	10	9.7	5.7	4.9	96.5

The method was validated for precision, accuracy, interday precision, intraday precision, and percent recovery (Section 2.9). Values are reported in Table 5.

The developed LC-MS/MS method corroborates our hypothesis (Fig. 6) that inhibition of the ADA enzyme or inability for it to metabolize Ado is essential in creating DBS QC material. To confirm this, we measured Ado and Ino in four DBS specimens (A–D), treated

under various conditions, enriched with equal concentrations of Ado: (A) 10  $\mu\text{M}$  enrichment of D-( $\beta$ )-Ado, 0  $\mu\text{M}$  EHNA (0.22  $\mu\text{M}$  Ado recovery), (B) 10  $\mu\text{M}$  enrichment of D-( $\beta$ )-Ado, 80  $\mu\text{M}$  EHNA (3.37  $\mu\text{M}$  Ado recovery), (C) 10  $\mu\text{M}$  enrichment of D-( $\alpha$ )-Ado, 80  $\mu\text{M}$  EHNA (5.77  $\mu\text{M}$  Ado recovery), and (D) 10  $\mu\text{M}$  enrichment of L-Ado, 0  $\mu\text{M}$  EHNA (9.98  $\mu\text{M}$  Ado recovery). As shown in Fig. 6, as Ado recovery increases, Ino production



**Fig. 6.** LC-MS/MS chromatograms of four DBS samples, enriched with 10  $\mu\text{M}$  Ado, showing the intensity of Ado and its metabolite, Ino. A) 10  $\mu\text{M}$  D-( $\beta$ )-Ado, 0  $\mu\text{M}$  EHNA (0.22  $\mu\text{M}$  Ado recovery); B) 10  $\mu\text{M}$  D-( $\beta$ )-Ado, 80  $\mu\text{M}$  EHNA (3.37  $\mu\text{M}$  Ado recovery); C) 10  $\mu\text{M}$  D-( $\alpha$ )-Ado, 80  $\mu\text{M}$  EHNA (5.77  $\mu\text{M}$  Ado recovery); and D) 10  $\mu\text{M}$  L-Ado, 0  $\mu\text{M}$  EHNA (9.98  $\mu\text{M}$  Ado recovery), using described HPLC-MS/MS method. As hypothesized, Ado is metabolized to Ino when the active ADA enzyme is present, as indicated by panels A-D.

decreases. Conversely, when lower Ado recoveries are observed the intensity of Ino is elevated.

#### 4. Discussion

Recovery of D-Ado and D-dAdo from DBS has previously been reported using the ADA inhibitor EHNA. Enrichment of blood with unnatural L-nucleosides represents an attractive alternative, as these compounds are naturally resistant to enzymatic degradation. This allows for the preparation of QC material requiring significantly less handling time, while decreasing the total cost of the preparation. This method demonstrates that L-Ado and L-2'-dAdo can act as reliable surrogates in the preparation of QC material for ADA-SCID NBS. Since these compounds are enantiomers of the target metabolites, their ionization properties and chromatographic performance (on an achiral column) are identical.

These analytes can be quantified in DBS using a 45-minute extraction with a range of solvent mixtures, a 1-minute inlet method flow injection analysis with binary solvents, and positive ion mode MS/MS analysis. These are the same conditions in which amino acids, acylcarnitines, and succinylacetone are screened and these metabolites experienced no significant change in reported concentration with the addition of the Ado and dAdo SRMs, as well as with the addition of  $^{13}\text{C}_5$ -Ado and  $^{13}\text{C}_5$ -dAdo to the internal standard mixture.

An LC-MS/MS method was also described for the separation of Ado and 2'-dAdo from their metabolites, Ino and 2'-dIno. Recovery of L-2'-dAdo was consistently > 90% in nearly all QC material while L-Ado was slightly lower at 80–85%. Both of these recoveries were consistently higher than those achieved through EHNA inhibition

of ADA in our hands. The current enrichment method will be used soon to generate DBS based quality assurance materials (for quality control and proficiency testing) in an ISO 17043-accredited laboratory to assist newborn screening programs. Those materials will be available upon request from the Newborn Screening and Molecular Biology Branch of the Centers for Disease Control and Prevention. The Newborn Quality Assurance Program (NSQAP) is designed to assist public health laboratories maintain and enhance the quality of newborn screening test results. The production of this material, and its distribution, will assure that the translation of methods into laboratory tests leads to sustainable high-quality testing and healthier babies worldwide.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

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