ANALYSIS OF ANIONS AND PROTEINS RELATED TO OXALATE METABOLISM IN HUMAN ERYTHROCYTES

By

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A Thesis Submitted to the Graduate Faculty of

WAKE FOREST UNIVERSITY GRADUATE SCHOOL OF ARTS AND SCIENCES

In Partial Fulfillment of the Requirements

For the Degree of

Master of Science

In the Molecular Genetics and Genomics Program

May 2009

Winston-Salem, North Carolina

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ACKNOWLEDGEMENTS

I would first like to thank my mentor, Dr. Ross Holmes, for providing me with research support, for sharing with me his wealth of knowledge, for his patience in training me, and for his guidance while allowing me the freedom to pursue my scientific interests. I would also like to thank Dr. John Knight for spending countless hours training me on the mass spectrometer and for sharing his wisdom of graduate student life - you offered me your support and guidance from the very start and for that I am grateful. I would like to thank the Ronald E. McNair Fellowship Program at Louisiana State University, without which I might never have had the opportunity to pursue a graduate degree. Thank you to my family for their constant support and understanding throughout my college career, particularly my brother Travis for dispensing advice that can only be heard from an older sibling, my brother Randal for impressing upon me the importance of listening, and my sister Meghan for being my true inspiration to study the sciences. Thank you to my professors for challenging and motivating me - particularly Dr. Dwayne Godwin. Thank you to my fellow classmates and friends, especially Hetal Pandya and Deon Edgerson, for listening, caring, and offering advice regardless of the time or place. Lastly, I would like to thank my mother and father for pushing me to succeed and to live up to my full potential. You are both truly gifted and loving parents and I couldn't have asked for better teachers.

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ABBREVIATIONS

GR/HPR - Glyoxylate reductase/hydroxypyruvate reductase (refers to protein)

GR - Glyoxylate reductase (refers to protein activity)

PH2 - Primary hyperoxaluria type 2

LDH - Lactate dehydrogenase

AGT - Alanine:glyoxylate aminotransferase

PH1 - Primary hyperoxaluria type 1

GO - Glycolate oxidase

DAO - D-amino acid oxidase

GCS - Glycine cleavage system

GOA - Glutamate:oxaloacetate aminotransferase

HKGA - 4-hydroxy-2-ketoglutarate lyase

SHMT - Serine hydroxymethyltransferase

THF - Tetrahydrofolate

ESWL - Extracorporeal shock wave lithotripsy

BMC - Blood mononuclear cells

RBC - Red blood cells

DPG - 2,3-diphosphoglycerate

DPGM - Diphosphoglycerate mutase

PG - 2-phosphoglycerate

PGP - Phosphoglycolate phosphatase

MCT - Monocarboxylic acid transporter

IRB - Institutional Review Board

DGDH - D-glycerate dehydrogenase

PBS - Phosphate buffered saline

TCA - Trichloroacetic acid

IC/MS - Ion chromatography coupled with mass spectrometry

ECD - Electrochemical detector

MD - Matrix diversion

ESI - Electrospray ionization

LOD - Limit of detection

PH - Phenylhydrazine

WT - Wild-type

KO - Knock-out

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

SIM - Single ion monitor

DBS - Dried blood spot

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ANALYSIS OF ANIONS AND PROTEINS RELATED TO OXALATE METABOLISM IN HUMAN ERYTHROCYTES

Thesis under direction of Ross P. Holmes, Ph.D., Professor of Urology

ABSTRACT

The metabolism of oxalate and its related anions in human erythrocytes have not been previously studied in detail. In this study, we identified the presence and investigated the functional role of glyoxylate reductase/hydroxypyruvate reductase (GR/HPR), a key protein in the metabolism of oxalate, in human erythrocytes. In addition, the presence of anions associated with oxalate metabolism was investigated using a novel technique that couples ion chromatography and mass spectrometry to reveal anions that previously eluded scientists due to their co-elution. Erythrocyte incubations with glycolate, glyoxylate, and C-13 glycolate were used to study whether these acids were precursors of oxalate or phosphoglycolate. This study also assessed a more cost-effective and less-invasive diagnostic assay called Dried Blood Spot Filter Paper Assay for the detection of Primary Hyperoxaluria Type 2 (PH2), a disease characterized by the dysfunction of GR/HPR. GR/HPR activity was detected in erythrocytes at levels similar to other blood components such as blood mononuclear cells. Anions associated with GR/HPR and oxalate metabolism were detected and quantified in 30 normal subjects. Erythrocyte incubations with glyoxylate revealed that elevated intracellular levels of glyoxylate produced elevated intracellular levels of oxalate. Erythrocyte incubations with glycolate showed that there was no significant increase in intracellular oxalate or phosphoglycolate levels as intracellular glycolate levels increased. The analysis of a

follow-up study using C-13 glycolate erythrocyte incubations was unable to reveal the metabolic fate of glycolate in erythrocytes. The assessment of the Dried Blood Spot Filter Paper Assay identified the assay as a promising tool for the diagnosis of PH2. In conclusion, this study investigated a previously un-described metabolic pathway in human erythrocytes. Further investigation of this pathway could lead to a greater understanding of oxalate metabolism, better diagnostic assays, and superior treatment strategies for PH2.

INTRODUCTION

Properties of oxalate. Oxalate is a dicarboxylic acid that acts as a chelator of various positively charged metals, such as sodium and calcium[1]. It is naturally occurring in plants and animals, and humans may acquire oxalate through their diet or through the metabolic catalysis of hydroxyproline, glycine, and other metabolites. Oxalate metabolism is especially important in patients with primary hyperoxaluria[2]. Calcium oxalate crystal deposition may develop in many tissues of the body, but it primarily occurs in the kidneys.

Clinical significance. When calcium oxalate crystal deposition occurs in the urine, kidney stones may form. Over 80% of all kidney stones are comprised of calcium oxalate[3], and each year, there are approximately 1.2 million new kidney stone cases resulting in $2.07 billion expended annually in the United Stated for their evaluation and treatment[4]. In fact, 5.2% of adults in the U.S. have had at least one kidney stone in their lifetime.

Oxalate metabolism. Calcium oxalate crystal formation occurs primarily in the kidneys and relies heavily on the synthesis of oxalate in the liver[5]. A summary of the pathways associated with oxalate synthesis in hepatocytes was published by Holmes in 2004 and can be found in Figure 1[6]. The major precursors of oxalate in the liver are glyoxylate and glycolate[6]. Glyoxylate is produced from the catabolism of hydroxyproline, glycine, and other metabolites, and typically, the bulk of glyoxylate is converted to glycolate via glyoxylate reductase/hydroxypyruvate reductase (GR/HPR) in the mitochondrion[5]. When a mutation in the coding region of GR/HPR that affects the activity of the enzyme occurs, a build up of glyoxylate results, and this excess is

converted to oxalate by lactate dehydrogenase (LDH) and glycolate oxidase (GO)[7]. Over a dozen of these mutations have been identified and characterized in a monogenic disease known as Primary Hyperoxaluria Type 2 (PH2). PH2 disease is marked by excessive oxalate and L-glycerate in the urine. Alanine:glyoxylate aminotransferase (AGT), another major protein in oxalate metabolism, is responsible for the conversion of glyoxylate to glycine. When a mutation occurs in the coding region of AGT that results in a decrease in enzymatic activity, a buildup of glyoxylate results and this excess is converted to oxalate by lactate dehydrogenase (LDH) and glycolate oxidase (GO)[7]. Some of these mutations have also been identified and characterized in a monogenic disease known as Primary Hyperoxaluria Type 1 (PH1)[8]. PH1 disease is marked by excessive oxalate and glycolate in the urine. In both PH1 and PH2 disease, oxalosis of the major organ systems occurs particularly in the kidneys where calcium oxalate stones form[9].

In addition to protein abnormalities, other causes of oxalosis include gastric bypass surgery and the inhibition of intestinal lipases by Orlistat[10,11]. Gastric bypass surgery causes enteric hyperoxaluria by decreasing the fat and bile acid absorption in the intestine which causes calcium to bind with the excess of fatty acids rather than oxalate as in the normal state. Additionally, the increased fatty acids and bile in the intestine increase the colon's ability to absorb oxalate, thus resulting in hyperoxaluria. In the case of Orlistat, an intestinal lipase inhibitor used as an anti-obesity drug, the unabsorbed fatty acids bind with calcium in the intestine which limits the available calcium to bind with oxalate. In such cases, the oxalate nephropathy was not identified until after injury to the kidney occurred.

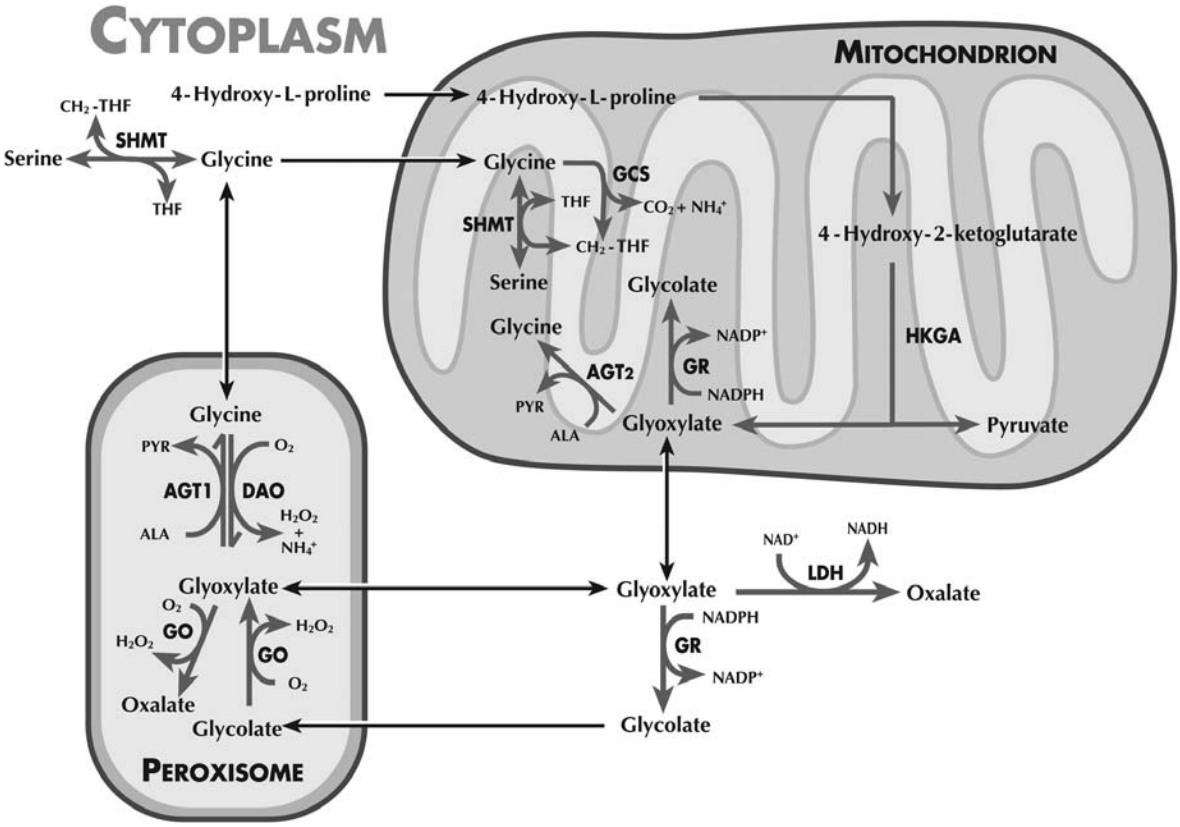


Figure 1. Pathways associated with oxalate synthesis in hepatocytes. Transport processes are shown as the thin, dark arrows and enzymatic reactions as the thicker, lighter arrows. DAO, D-amino acid oxidase; GCS, glycine cleavage system; GO, glycolate oxidase; GOA, glutamate:Oxaloacetate aminotransferase; GR, glyoxylate reductase; HKGA, 4-hydroxy-2-ketoglutarate lyase; LDH, lactate dehydrogenase; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate. Figure reproduced with permission from Baker et al. 2004[6].

The current treatment for the primary hyperoxalurias is essentially non-specific and includes a regimen to treat the multiple kidney stone formations. This regimen includes an increase in fluid intake, a modified diet, and extracorporeal shock wave lithotripsy (ESWL) to pulverize the stones and allow their expulsion into the urine. However, there is evidence that the concentrated shock waves in ESWL lead to permanent damage to healthy tissue in the kidney, and a 2006 study by the Mayo Clinic suggests that lithotripsy may increase subsequent incidence of diabetes and hypertension[4]. Some progress has been made in the treatment of PH1 disease with pyridoxine, a cofactor of AGT. Pyridoxine treatment decreases oxalate production in approximately 38% of PH1 patients with a specific AGT mistargeting mutation, of which the majority of patients have a partial response to treatment. In severe cases of primary hyperoxaluria, dialysis is used to filter out some of the excess metabolites for up to 8 hours/day or more, but ultimately, a kidney and/or liver transplant is required.

Properties of GR/HPR. The crystal structure of GR/HPR has been described at 2.2 Å resolution[12]. GR/HPR occurs as a dimer with substrate specificity for glyoxylate and hydroxypyruvate. The crystal structure also implicates NADPH as a necessary co-factor for GR activity. Researchers in this field have sequenced mutations in the gene regions of GR/HPR in an attempt to find more efficient diagnostic tools for the primary hyperoxalurias[13]. In a study by Rumsby *et al.* one common mutation in the coding region of GR/HPR was discovered. In a subsequent study by Webster *et al.*, five additional mutations were discovered[8]. Research has also been performed to identify the consequences of mutations. Such consequences may include intracellular mistargeting,

enzymatic deficiencies, and metabolic fluxes. In spite of the significant strides in hyperoxaluria research made in recent years, there is still much to be discovered.

Red cell oxalate metabolism. GRHPR has been detected in most body tissues, including blood mononuclear cells (BMC)[14]. This finding prompted the investigation of GRHPR in red blood cells (RBC). RBC are mainly responsible for the transport of oxygen to body tissues. These cells need to be able to adjust to various environmental levels of oxygen, for example, hypoxia due to an increase in atmospheric elevation[15]. RBC cope with these changes by utilizing a metabolic intermediate known as 2,3-diphosphoglycerate (DPG), which binds the deoxygenated form of hemoglobin and reduces its affinity for oxygen. This causes a structural change in the hemoglobin tetramer that favors oxygenation of tissues. The binding of 2,3-DPG is regulated by diphosphoglycerate mutase (DPGM), an erythrocyte-specific trifunctional enzyme with synthase activity that catalyzes the conversion of 1,3-DPG to 2,3-DPG, mutase activity that catalyzes the interconversion between 2- and 3-phosphoglycerate, and phosphatase activity that hydrolyzes 2,3-DPG to 2-phosphoglycerate (PG) and a phosphate[16]. 3-PG is a known inhibitor of DPGM's mutase activity, and phosphoglycolate is a potent activator of DPGM's phosphatase activity; however, the mechanism by which phosphoglycolate is produced is not completely understood[17]. It has been proposed that pyruvate kinase activity could contribute to phosphoglycolate levels, but scientists have disputed this claim[18]. Although the phosphatase activity of DPGM can be activated by a host of anions[17], phosphoglycolate is the most significant contributor to its activation, increasing its maximal velocity by 1600 times the enzyme rate in the absence of activators. The K_a for DPGM phosphatase activity activated by phosphoglycolate is 8μM[19].

The phosphoglycolate concentration in human erythrocytes has been previously recorded at 2.5-5μM[19] and phosphoglycolate phosphatase [PGP] activity may aid in the maintenance of these concentrations. PGP has six common isoforms and is activated by chlorides, inorganic acids, and carboxylic acids and inhibited by high levels of phosphoglycolate[20,21]. The human PGP gene and its associated polymorphisms have been extensively studied[21], and many hypotheses regarding the presence and metabolic significance of phosphoglycolate have been made. This finding suggests that other proteins that contribute to phosphoglycolate level maintenance exist. Further, the metabolic source for glycolate, a precursor of phosphoglycolate, must also be identified. One potential source of glycolate in erythrocytes is the conversion of glyoxylate to glycolate by GR/HPR. Glycolate may also be imported from the plasma through the monocarboxylic acid transporter (MCT) type 1[22]. Red cell membranes have been found to be more permeable to glycolate than glyoxylate.

Methods of anion measurement. In addition to identifying the sources of phosphoglycolate and glycolate, another factor that has impeded research of oxalate metabolism is the lack of an efficient and reliable method of detecting metabolites involved in oxalate metabolism. Much of the controversy surrounding the presence of phosphoglycolate in human erythrocytes seems to lie in the ability to detect erythrocyte metabolic constituents accurately. Up to this point, enzymatic assays developed by various analytical supply companies have been utilized to detect 2- and 3-phosphoglycerate and 2,3-diphosphoglycerate, but these assays have limitations in their sensitivity and specificity because they do not directly measure the associated metabolites[23]. Phosphoglycolate has been measured using a complicated and time-

consuming method involving multiple passes on a Dowex 1-Cl^- column and various enzymatic assays to gather data correction information[22]. To date, this is still the only published method used to directly measure phosphoglycolate.

In the Holmes laboratory, a method that utilizes the coupling of ion chromatography with mass spectrometry, has allowed the direct measurement of phosphoglycolate and oxalate-associated anions. Scientists are now able to detect anions that typically co-elute using conventional ion chromatography. This newly-developed method arms researchers with a powerful tool to investigate oxalate metabolism.

METHODS

Human Subjects. Participants included 14 male and 16 female healthy, non-stone forming subjects at Wake Forest University Baptist Medical Center (median age 28yrs, range 23yrs-53yrs) with no prior history of metabolic disorders. Written consent was given by all subjects and a questionnaire concerning their general medical history was completed. This study was approved by the Institutional Review Boards (IRB) of Wake Forest University Health Sciences and informed, written consent was received by all subjects. The IRB designated this study number IRB00002973.

Animals. The GR/HPR knockout mice used in this study are the result of a cross between the 129SvEvBrd and C57Bl/6 strains which have a null Grhpr allele. Animals were housed in the Animal Resources Program facility at Wake Forest University Health Sciences. Mice were fed standard diets and blood was drawn by buccal lancet puncture. The GR/HPR knockout mice exhibit hyperoxaluria, undetectable D-glycerate dehydrogenase (DGDH) activity, and elevated plasma oxalate and glycerate.

Blood Collection and Preparation. Two fasted blood samples (approx. 6 ml each) were collected into pre-chilled, BD Vacutainer^{TM} Sodium Heparin tubes (Becton Dickinson & Company, NJ). A second fasted blood sample was obtained one week later at the same time of day. Six participants were re-sampled one year after the initial blood draw. Blood was placed on ice immediately after blood draw and processed within 6 hours. Overall anion change during six hours on ice was $\leq 5\%$. Red cells were separated by centrifugation at 1,500 rcf for 5 min at 4ºC. Plasma and buffy coat were suctioned and red cells were washed three times with 1X Phosphate Buffered Solution (PBS), pH 7.4, by vortexing, centrifuging at 1,500 rcf for 5 min at 4ºC, and suctioning the supernatant

after each spin. No hemolysis of red cells was observed during this process. After final wash and removal of supernatant, 200µl of packed red cells was transferred into a pre-chilled Eppendorf tube by using a positive-displacement pipette for precision. The remaining red cells were stored at -70ºC.

Hemoglobin Analysis. The Drabkin's Assay was used for hemoglobin analysis.[24] Drabkin's Reagent (200mg/L $K_3Fe(CN)_6$, 1g/L $NaHCO_3$ (pH 8.6), 50mg/L KCN) was added to whole blood samples converting hemoglobin to cyanomethemoglobin, which has an absorbance at 540nm. Cyanomethemoglobin has a broad absorption peak that allows its measurement between 530 and 550 nm. A standardization curve was generated by using lyophilized human hemoglobin from Sigma-Aldrich, Inc (St. Louis, MO).

Anion Analysis. Red cells proteins were precipitated using 10% ice-cold trichloroacetic acid (TCA) for 20 min. The acid was removed by vigorously vortexing for 4 min with an equal volume of 1,1,2-trichlorotrifluorethane(Freon)-trioctylamine (3:1 v/v; Aldrich, Milwaukee, WI, USA) followed by centrifuging at 4ºC. to separate the upper aqueous layer[25]. The aqueous layer was removed and analyzed by ion chromatography coupled with a single quadrapole mass spectrometer depicted in Figure 2 (IC/MS; Dionex Corporation, Sunnyvale, Calif., USA). The ion chromatography equipment consisted of a Dionex ED50 conductivity detector, Dionex AS11_HC 2 x 250 mm ion exchange column (packed with a highly cross-linked core made of 13µm microporous resin beads and a MicroBead® anion-exchange layer attached to the surface) with guard column at a controlled temperature of 30 ° C, and a Dionex ASRS-ULTRA 2-mm suppressor. A sodium hydroxide gradient 1–40 mM over 65 min at a flow rate of 0.4 ml/min was used. Dilutions of samples were made to account for the varying

concentrations of the following anions: α-ketoglutarate, 2,3-diphosphoglycerate, D-glycerate, glycolate, lactate, 3-phosphoglycerate, phosphoenolpyruvate, phosphoglycolate, pyruvate, citrate, fructose-6-phosphate, fumarate, galactose-6-phosphate, glucose-6-phosphate, and mannose-6-phosphate. The limit of detection (LOD) was determined by calculating the mean blank signal and adding ten times the standard deviation. The LOD for these anions was 0.16μM, 0.20μM, 0.11μM, 0.04μM, 0.07μM, 0.07μM, 0.05μM, 0.07μM, 0.05μM, 0.01μM, 4.24μM, 0.02μM, 0.07μM, 0.09μM, and 0.44μM respectively.

Glyoxylate & Hydroxypyruvate Analysis. Red cell proteins were precipitated with 10% perchloric acid [PCA] for 20 min. Precipitates were centrifuged at 13,200 rpm for 5 min. and derivatized for 15 min. with 0.1% phenylhydrazine. Samples were analyzed via reversed-phase HPLC as in Figure 3. Samples were injected onto a DYNAMAX C18 HPLC Column (4.6 x 150-mm) and separated at a flow rate of 1.0ml/min. Peaks were detected at a wavelength of 313nm with a BIORAD UV Monitor and results were analyzed with EmpowerPro Version 5. The limit of detection (LOD) was determined by calculating the mean blank signal and adding ten times the standard deviation. The LOD for glyoxylate and hydroxypyruvate were 0.04μM and 0.18μM respectively.

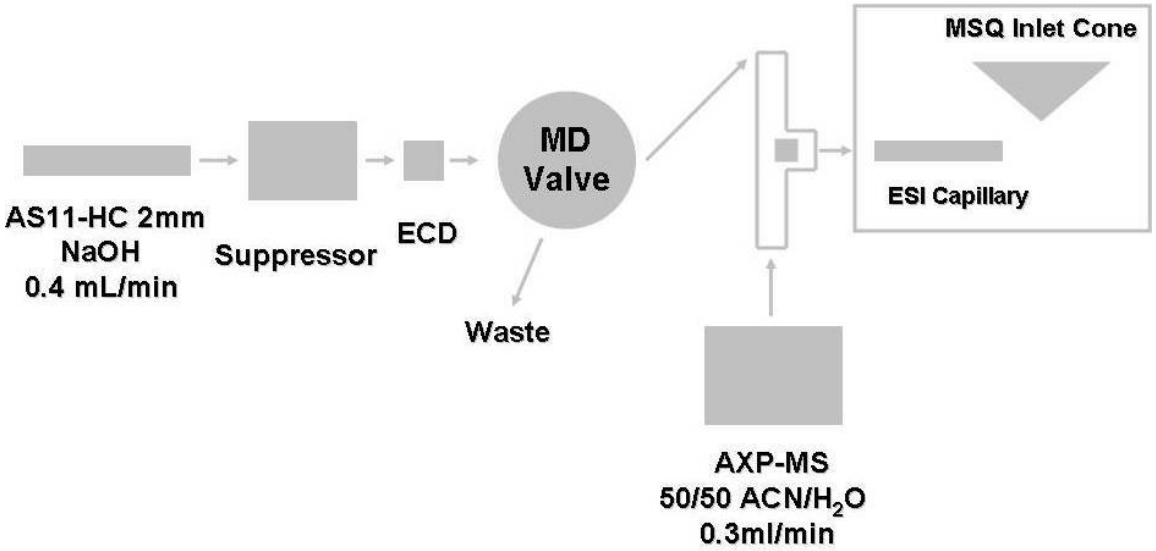


Figure 2. Flow diagram of IC/MS components with matrix diversion. A sample is introduced into a column that contains a stationary phase (AS11-HC 2mm). A mobile phase carries the sample through the column (NaOH). The analytes are retained on the stationary phase and can be eluted by increasing the concentration of a similar charge that will displace the analyte ions from the stationary phase. Following this separation, samples are filtered through a suppressor to reduce background eluent noise signal and increase analyte signal. Analytes are then directed through the electrochemical detector (ECD) where a signal is generated based on the time a sample exits the system. Next a matrix diversion (MD) valve controls when analytes pass into the mass spectrometer or are diverted to a waste container in order to minimize the mass spectrometer's exposure to high levels of analytes. An electrospray ionization (ESI) capillary delivers the sample to be measured past the MSQ inlet cone where a vacuum carries the sample into the RF lens, the single quadropole mass analyzer, and then the detector, which converts the measured analyte fragments to a signal based on their mass:charge ratio.

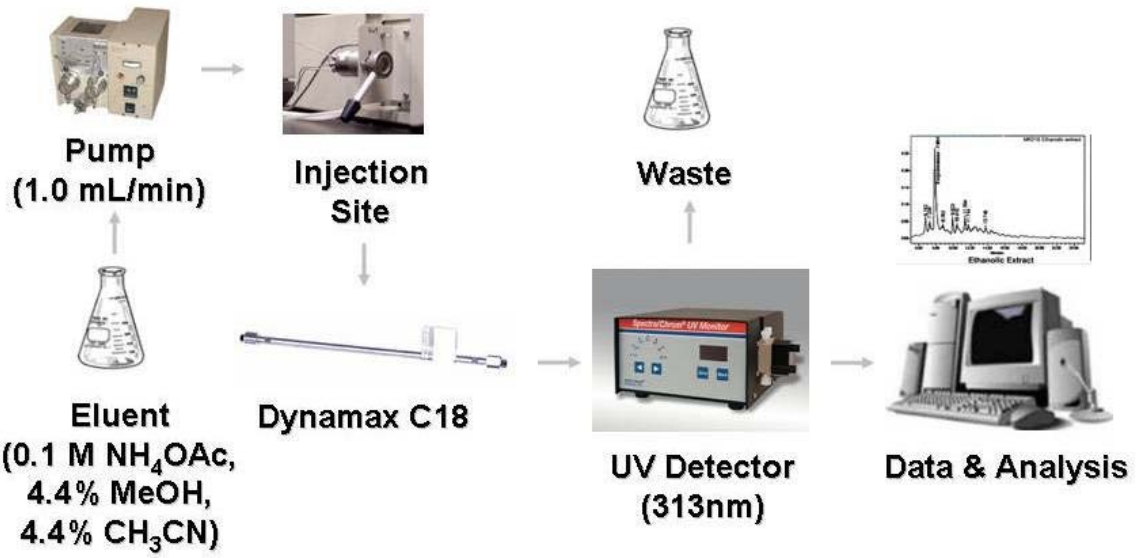


Figure 3. Flow diagram of HPLC components. A sample is introduced into a Dynamax C18 column at the injection site. An isocratic mobile phase (0.1M NH_4OAc, 4.4% MeOH, 4.4% CH_3CN) carries the sample through the column and the carboxyllic acids are retained on the stationary phase based on their polarity. Following this separation, samples enter a UV Detector where their signal is recorded and transmitted to a computer for analysis. Photos from Waters, Varian, and BioRad.

D-glycerate Dehydrogenase (DGDH) Activity Detection. As noted previously, the concentrations of hemoglobin were calculated in whole blood samples, but DGDH activity was measured in RBC. In order to account for this difference, 100µl of whole blood was aliquotted and washed three times in PBS. Special care was taken to remove the least amount of RBC. By using this method, we were able to determine activity in RBC and still express its activity in units per mg hemoglobin. DGDH activity was measured as previously [26] with hydroxypyruvate production detected by HPLC following a 20 minute assay and 15 minute phenylhydrazine (PH) incubation. In PH incubations, α-keto acids are derivatized by PH which has an absorbance of 313nm. This enables the detection of phenylhydrazones formed via UV spectrophotometry. The DGDH assay consisted of 20mM Tris (pH 8.5), 20mM D-Glycerate, and 1mM NADP. Purified RBC samples were lysed 1:10 with 25mM HEPES and 0.1% Triton X-100. The DGDH assay was terminated by incubating with 1M perchloric acid after 20 minutes in a 37°C water bath. Centrifugation was used to remove protein precipitates and a 1mM phenylhydrazine 15 minute incubation was used to label α-keto acids.

Glyoxylate Reductase/Hydroxypyruvate Reductase (GR/HPR) Analysis. The GR/HPR assay was modified from a previously run assay [26] which measured glycolate production of a 5 minute incubation in a 37°C water bath with 50mM HEPES, 100mM KCl, 0.2mM NADPH, and 6mM Glx. In this experiment, glycolate was measured by IC coupled with electrospray single quadropole mass spectrometry detection (IC-MS) [Dionex Corp., Sunnyvale, CA] because the hemoglobin in red cell lysates interfered with the spectrophotometric detection methods. The IC apparatus contained a Dionex ED50 conductivity detector, Dionex AS11-HC 2 x 250-mm ion exchange column

(packed with a highly cross-linked core made of 13μm microporous resin beads and a MicroBead® anion-exchange layer attached to the surface) with guard column at a controlled temperature of 30°C, and a Dionex ASRS-ULTRA 2-mm suppressor. A gradient of 0.5-1mM sodium hydroxide over 35 minutes at 0.4 ml/min flow rate was used to separate glycolate from other anions. The LOD for glycolate was 0.2μM.

Oxalate Analysis**.** Oxalate samples from red blood cells were prepared as in anion analysis, then 0.2M boric acid (pH 3.85) was added and samples were measured via IC using a standard curve. The IC apparatus used for oxalate analysis consisted of a Dionex GP50 gradient pump, AS50 auto sampler, ED50 electrochemical detector, AS4ASC 2 x 250-mm ion exchange column with an ASRS – ULTRA II 2mm self-regenerating suppressor which reduces excess mobile phase background anions thus increasing detection sensitivity of sample anions. The mobile phase was 1.8mM Na_2CO_3, 1.7mM $NaHCO_3$ at 0.5ml/min. The LOD for oxalate was 0.24μM.

Western Blot Analysis. RBC lysates were electroblotted from 12% (w/v) acrylamide gels onto Hybond-C nitrocellulose membranes (Amersham Biosciences, Piscataway, N.J., USA). The membranes were incubated overnight in 5% (w/v) nonfat dried milk, PBS (pH 7.4), and 1% (w/v) Tween-20 at 4°C. Rabbit antirecombinant human GRHPR (hGRHPR) antibody (1μg/ml) was applied to the membrane for 1 hour at RT. The membrane was then washed and incubated a second time with peroxidase-conjugated goat antirabbit immunoglobin G (0.2μg/ml; Dako Corporation, Carpentaria, CA, USA) for 1 hour at RT. The bands were visualized by enhanced chemiluminescence (Amersham Biosciences) and films were analyzed with ImageJ 1.36b Software (Wayne Rasband, National Institutes of Health, USA).

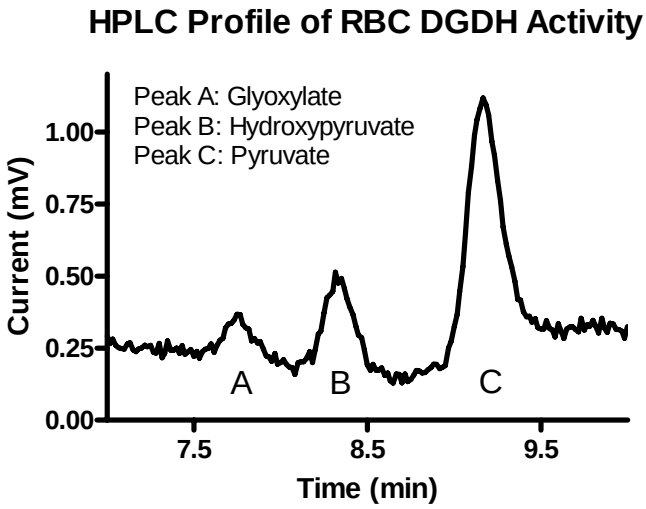
DBS Analysis. Whole blood from a finger prick was dropped directly onto Whatman ProteinSaver 903® filter paper (Schleicher & Schuell, Florham Park, New Jersey). Whatman filter paper was specially designed for the Dried Blood Spot Assay that is typically performed on infant heel sticks to detect a range of diseases including congenital hypothyroidism, galactosemia, branched-chain ketonuria, maple sugar urine disorder and sickle-cell anemia. After drying blood spots for 4 hours at RT, 16mm pre-printed circles were cut from the membrane, sliced, and placed into a 1.7ml Eppendorf tube. A blood spot of 16mm absorbed approximately 50µl blood. Blood was extracted from filter paper by adding 450µl 0.1% Titon X-100 and incubating on ice for 10 min. Blood samples were then vortexed for an additional 10 min at RT. Lysates were then used for enzymatic and metabolite analysis.

RESULTS

DGDH & GR activity in human and mouse erythrocytes. Both human and wild type mouse erythrocytes have DGDH activity, exemplified in Figure 4. Figure 4(A) displays the HPLC profile of the hydroxypyruvate that is produced from the metabolism of D-glycerate when incubated with erythrocyte lysates. Figure 4(B) shows the marked decrease in D-glycerate metabolism in knock out mouse erythrocyte lysates when compared to wild type mouse erythrocyte lysates. GR/HPR assays were also run to confirm the implications made by the DGDH assay results. Table 1 contains a quantitative comparison of DGDH activities along with their respective GR/HPR activities. In Table 1(A), RBC lysate activity is compared to that of BMC's and recombinantly-expressed GR/HPR. RBC and BMC showed similar activity levels for both DGDH and GR/HPR activity. On average, GR/HPR activity was 10-fold higher than DGDH activity. This same 10-fold difference is seen when a comparison of the DGDH and GR/HPR activity of recombinantly-expressed GR/HPR is made. Table 1(B) compares the GR/HPR activity of wild-type and knock-out mouse erythrocytes. The DGDH activity of WT mouse RBC was significantly higher than the DGDH activity of the KO mouse RBC.

GR/HPR protein in human and mouse erythrocytes. Western blot analysis was performed to confirm the presence of GR/HPR in human and mouse erythrocytes. Figure 5 shows the Western blot comparison of recombinantly expressed GR/HPR with a band of similar size and immunoreactivity in human erythrocytes, wild-type mouse liver and erythrocyte lysates. This band was not observed in the erythrocytes of a PH2 patient or liver and erythrocytes of knock-out mice.

(A)



(B)

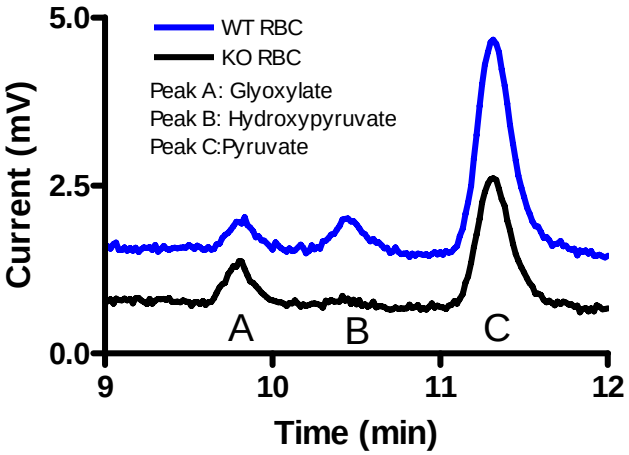


Figure 4. HPLC profile of DGDH activity and IC/MS profile of GR activity. (A) HPLC Profile of Human RBC DGDH Reaction: DGDH activity was measured by detecting hydroxypyruvate production using HPLC following a 20 minute assay and 15 minute phenylhydrazine (PH) incubation. The DGDH assay consisted of 20mM Tris (pH 8.5), 20mM D-Glycerate, and 1mM NADP. Purified RBC samples were lysed 1:10 with 25mM HEPES and 0.1% Triton X-100. The DGDH assay was terminated by incubating with 1M perchloric acid after 20 minutes in a 37°C water bath. Centrifugation was used to remove protein precipitates and a 1mM phenylhydrazine 15 minute incubation was used to label α-keto acids. DGDH activity is indicated by an increase in Peak B (hydroxypyruvate production after incubation with D-glycerate) (B) HPLC Profile of Wild-Type (WT) and Knock-Out (KO) Mouse RBC: DGDH activity is indicated by an increase in Peak B (hydroxypyruvate production after incubation with D-glycerate). The WT Mouse RBC showed DGDH activity while the KO Mouse RBC did not contain DGDH activity.

Table 1. DGDH and GR/HPR Activities in Humans and Mice.

(A)

	DGDH Activity (1 unit activity = 1 nmol hydroxypyruvate/mg total protein*min)	GR/HPR Activity (1 unit activity = 1 nmol glycolate/mg total protein*min)
RBC*	1.6 ± 0.57	19 ± 9.4
BMC	0.97 ± 0.20	11 ± 3.3
Recombinant GR/HPR	1400 ± 170	19000 ± 1500

(B)

	DGDH Activity (1 unit activity = 1 nmol hydroxypyruvate/mg total protein*min)
$GR/HPR^{+/+}$ (WT)Mouse RBC*	1.7 ± 0.44
$GR/HPR^{-/-}$ (KO) Mouse RBC*	0.35 ± 0.11

DGDH activity was measured by detecting hydroxypyruvate production using HPLC following a 20 minute assay and 15 minute phenylhydrazine (PH) incubation. The DGDH assay consisted of 20mM Tris (pH 8.5), 20mM D-Glycerate, and 1mM NADP. Purified RBC samples were lysed 1:10 with 25mM HEPES and 0.1% Triton X-100. The DGDH assay was terminated by incubating with 1M perchloric acid after 20 minutes in a 37°C water bath. Centrifugation was used to remove protein precipitates and a 1mM phenylhydrazine 15 minute incubation was used to label α-keto acids. GR/HPR activity was measured via glycolate production after a 5-minute incubation in a 37°C water bath with 50mM HEPES, 100mM KCl, 0.2mM NADPH, and 6mM Glx. In this experiment, glycolate was measured by IC coupled with electrospray single quadropole mass spectrometry detection (IC-MS). A gradient of 0.5-1mM sodium hydroxide over 35 minutes at 0.4 ml/min flow rate was used to separate glycolate from other anions. The LOD for glycolate was 0.2μM. (A) Comparison of DGDH and GR/HPR Activities in Human RBC and BMC and Comparison of Recombinant GR/HPR DGDH and GR/HPR Activities: RBC and BMC showed similar activity levels for both DGDH and GR/HPR activity. On average, GR/HPR activity was 10-fold higher than DGDH activity. This same 10-fold difference is seen when a comparison of the DGDH and GR/HPR activity of Recombinant GR/HPR is made. * For RBC, 1 unit activity = 1 nmol product/mg Hgb*min. For BMC and Recombinant GR/HPR, 1 unit activity = 1 nmol product/mg total protein*min (B) GR/HPR Activities in Mouse RBC: The DGDH activity of the WT Mouse RBC was significantly higher than the DGDH activity of the KO Mouse RBC. (1 unit activity = 1 nmol product/mg Hgb*min)

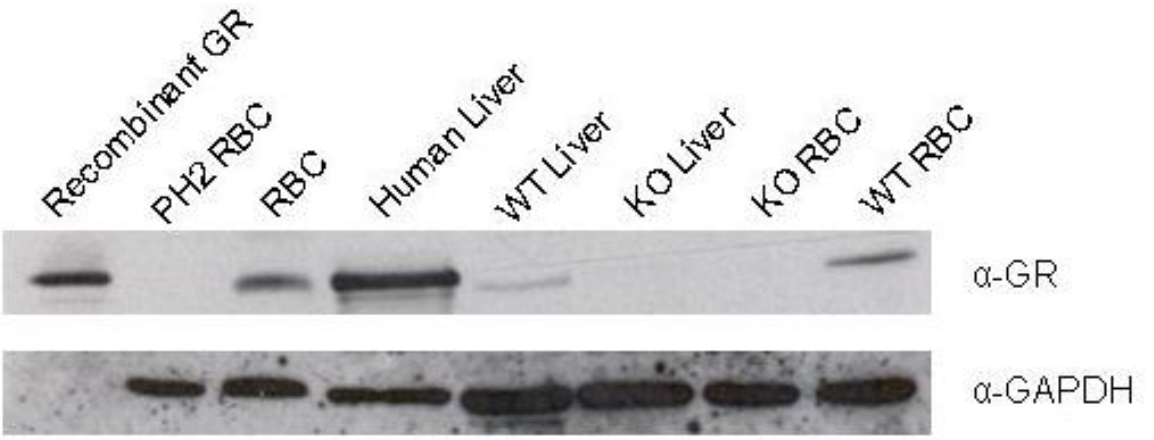


Figure 5. GR/HPR western blot. RBC lysates were electroblotted from 12% (w/v) acrylamide gels onto Hybond-C nitrocellulose membranes. The membranes were incubated overnight in 5% (w/v) nonfat dried milk, PBS (pH 7.4), and 1% (w/v) Tween-20 at 4°C. Rabbit antirecombinant human GRHPR (hGRHPR) antibody (1μg/ml) was applied to the membrane for 1 hour at RT. The membrane was then washed and incubated a second time with peroxidase-conjugated goat antirabbit immunoglobin G (0.2μg/ml) for 1 hour at RT. The bands were visualized by enhanced chemiluminescence and films were analyzed with ImageJ 1.36b Software. GR/HPR Construct, Human RBC, WT Mouse RBC, KO Mouse RBC, PH1 patient RBC, PH2 Patient RBC are shown with GR (top line) and GAPDH (glyceraldehyde 3-phosphate dehydrogenase; bottom line) blot overlay. GAPDH is a metabolic protein that is used as a marker for protein loading. Recombinant GR/HPR was used as a standard in this western blot. Recombinant GR/HPR shows the presence of GR/HPR and absence of GAPDH, because it is a purifed, recombinantly-expressed protein. All other samples show a presence of GAPDH. PH2 RBC shows an absence of GR/HPR. Human RBC shows the presence of GR/HPR. Human liver shows the presence of GR/HPR. WT Mouse liver shows the slight presence of GR/HPR. KO Mouse liver shows the absence of GR/HPR. KO Mouse RBC shows the absence of GR/HPR. WT Mouse RBC shoes the presence of GR/HPR

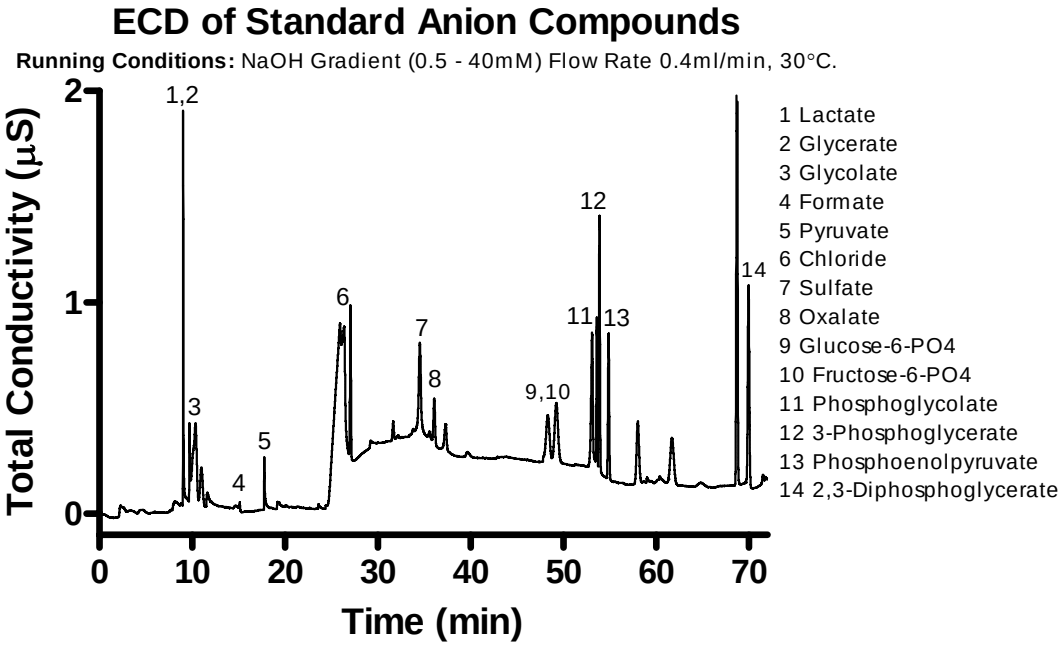
Anions associated with oxalate metabolism in human erythrocytes. Anions associated with oxalate metabolism are those anions that lead to or result from its metabolism. This also includes co-factors of enzymes associated with oxalate metabolism and the anions that lead to or result from co-factor metabolism. Figure 6(A) exhibits the standard profile of standard concentrations of the ions associated with oxalate metabolism. Figure 6(B) shows this profile in human erythrocyte lysates. Figure 7 demonstrates the power of coupling ion chromatography with mass spectrometry by revealing distinct peaks for anions that notoriously co-elute using ion chromatography alone.

Detecting anions within their linear range is typically a concern when utilizing mass spectrometry to study anion concentrations. Figure 8 includes standard concentration curves for the anions associated with oxalate metabolism. Each graph component displays the increase in peak values as concentration is increased. The curves include concentrations of 0.0625µM, 1.25µM, 2.5µM, 5µM, 10µM, and 20µM along with the R^2 value for best fit linearity. None of the R^2 values fell below 0.9916.

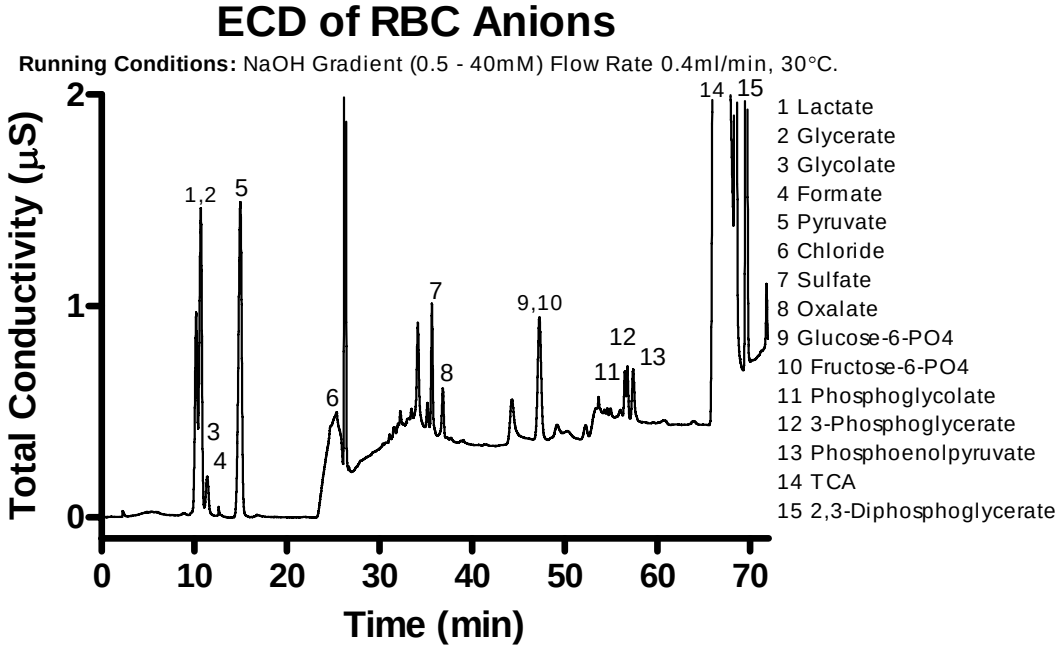
RBC anion stability during preparation and storage. Maintaining anion stability during the storage of erythrocyte lysates is crucial when developing diagnostic assays and investigating the role of metabolic anions. Figure 9 compares the efficiency of three standard methods of erythrocyte storage at -70ºC: washed and packed erythrocytes, TCA extracts, and neutralized TCA extracts.

Figure 6. IC output. Anions were detected using ion chromatography coupled with single quadrapole mass spectrometry. The ion chromatography equipment consisted of a Dionex ED50 conductivity detector, Dionex AS11_HC 2 x 250 mm ion exchange column (packed with a highly cross-linked core made of 13μm microporous resin beads and a MicroBead® anion-exchange layer attached to the surface) with guard column at a controlled temperature of 30 ° C, and a Dionex ASRS-ULTRA 2-mm suppressor. A sodium hydroxide gradient 1–40 mM over 65 min at a flow rate of 0.4 ml/min was used. Dilutions of samples were made to account for the varying concentrations of anions (A) ECD output of IC profile of standard anions at a 5μM concentration. All anions are not resolved, as this is only the IC profile. Anion peaks are resolved after mass spectrometry separation. (B) ECD output of IC profile for PCA-extracted erythrocytes diluted 1:30.

(A)


ECD of Standard Anion Compounds
Running Conditions: NaOH Gradient (0.5 - 40mM) Flow Rate 0.4ml/min, 30°C.
Total Conductivity (μS)
Time (min)
1 Lactate
2 Glycerate
3 Glycolate
4 Formate
5 Pyruvate
6 Chloride
7 Sulfate
8 Oxalate
9 Glucose-6-PO4
10 Fructose-6-PO4
11 Phosphoglycolate
12 3-Phosphoglycerate
13 Phosphoenolpyruvate
14 2,3-Diphosphoglycerate

(B)


ECD of RBC Anions
Running Conditions: NaOH Gradient (0.5 - 40mM) Flow Rate 0.4ml/min, 30°C.
Total Conductivity (μS)
Time (min)
1 Lactate
2 Glycerate
3 Glycolate
4 Formate
5 Pyruvate
6 Chloride
7 Sulfate
8 Oxalate
9 Glucose-6-PO4
10 Fructose-6-PO4
11 Phosphoglycolate
12 3-Phosphoglycerate
13 Phosphoenolpyruvate
14 TCA
15 2,3-Diphosphoglycerate

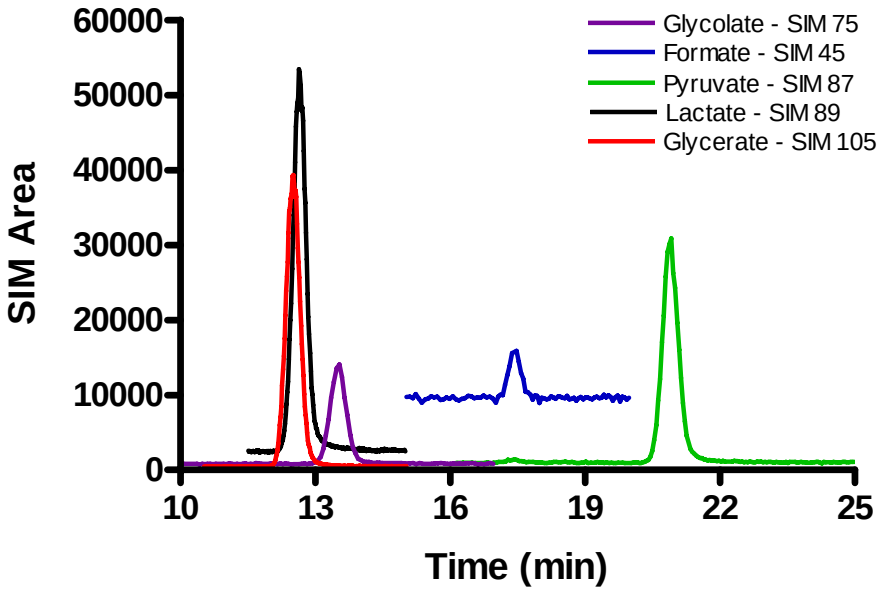


Figure 7. IC/MS output: Single Ion Monitor (SIM) analysis of selected compounds. Anions were detected using ion chromatography coupled with single quadrapole mass spectrometry. The ion chromatography equipment consisted of a Dionex ED50 conductivity detector, Dionex AS11_HC 2 x 250 mm ion exchange column (packed with a highly cross-linked core made of 13μm microporous resin beads and a MicroBead® anion-exchange layer attached to the surface) with guard column at a controlled temperature of 30 ° C, and a Dionex ASRS-ULTRA 2-mm suppressor. A sodium hydroxide gradient 1–40 mM over 65 min at a flow rate of 0.4 ml/min was used. Dilutions of samples were made to account for the varying concentrations of anions. This figure displays the SIM (single ion monitoring) output for anions that have been separated by the IC and then directed into the electrospray mass spectrometer. Anions are resolved by charge and mass. The SIM number for each anion is the anion's mass minus one mass unit.

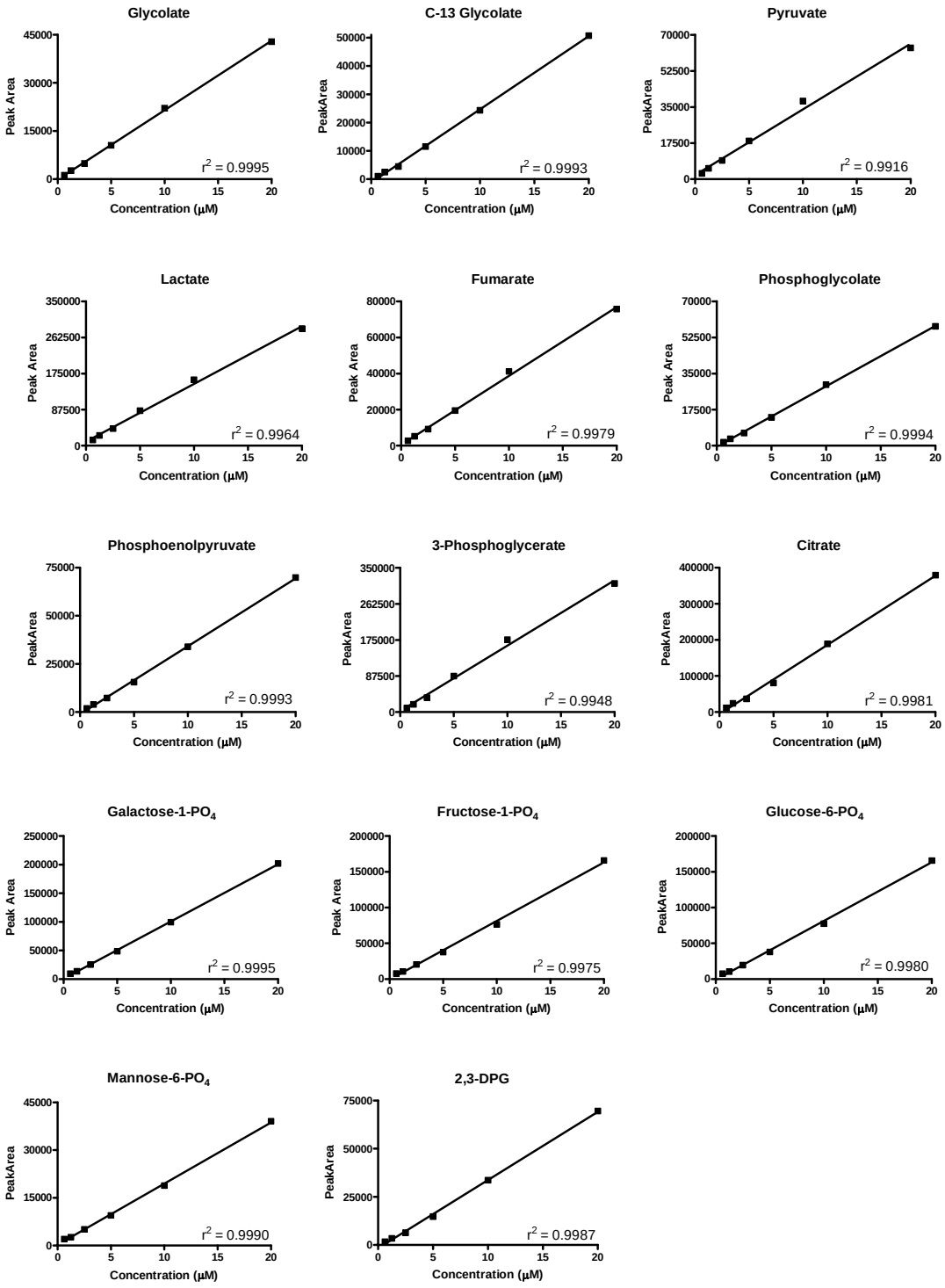
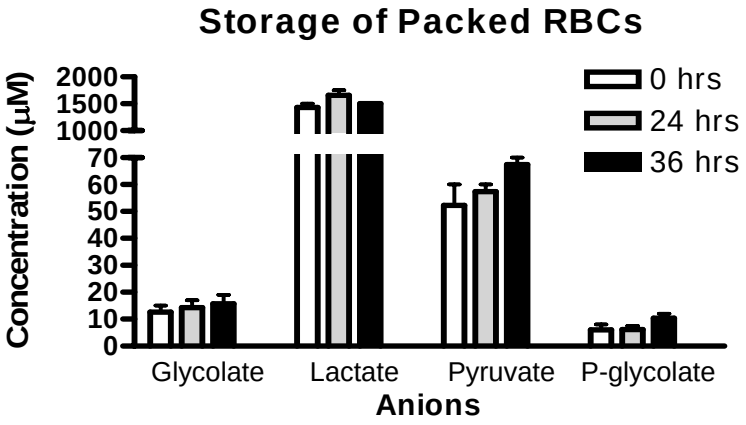
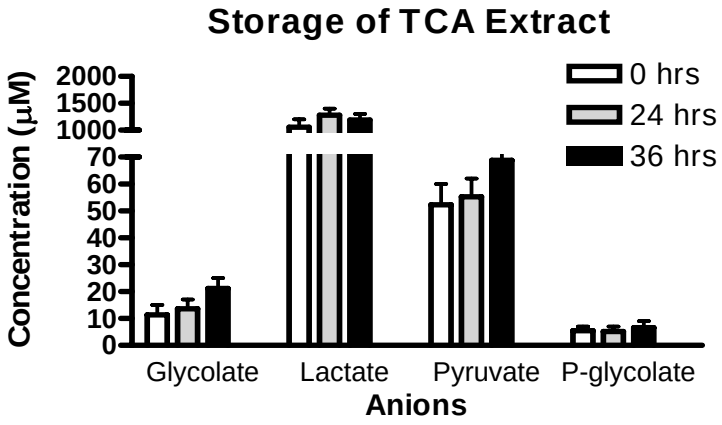
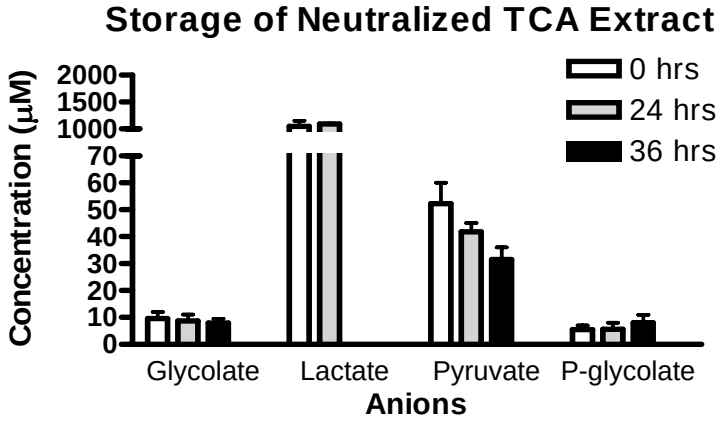


Figure 8. IC-MS Anion Standard Curves. 14 standard anion compounds were analyzed by creating peak area calibration curves to ensure detection of anions was within their linear range. Concentrations analyzed include 0.0625μM, 1.25μM, 2.50μM, 5.00μM, 10.0μM, and 20.0μM. Linear regression was calculated using GraphPad Prism® and r^2 values are displayed. Values for r^2 did not exceed 0.9916.

Figure 9. Erythrocyte Anion Levels at 24 and 36 Hours Using Various Storage Methods. Various methods of anion sample storage were compared including storage of packed red blood cells, storage of TCA anion extract, and storage of neutralized TCA anion extract. Packed red blood cells were prepared by separating the red cells from the plasma and buffy coat by centrifugation at 1,500 rcf for 5 min at 4ºC. Plasma and buffy coat were suctioned and red cells were washed three times with 1X Phosphate Buffered Saline (PBS), pH 7.4, by vortexing, centrifuging at 1,500 rcf for 5 min at 4ºC, and suctioning the supernatant after each spin. TCA extracts were prepared by precipitating the protein using 10% ice-cold trichloroacetic acid (TCA) for 20 min. Samples were then centrifuged at maximum speed for 5 min at 4ºC to remove the precipitated protein and the aqueous layer was extracted. The neutralized TCA extract was prepared by vigorously vortexing samples for 4 min. with an equal volume of 1,1,2-trichlorotrifluorethane(Freon)-trioctylamine (3:1 v/v; Aldrich, Milwaukee, WI, USA) followed by centrifuging at 4ºC. to separate the upper aqueous layer. In each of these methods, the sample was analyzed by ion chromatography coupled with a single quadrapole mass spectrometer. Each method was investigated in triplicate at 0, 24, and 36 hours.


Storage of Packed RBCs
Concentration (μM)
2000
1500
1000
70
60
50
40
30
20
10
0
0 hrs
24 hrs
36 hrs
Glycolate
Lactate
Pyruvate
P-glycolate
Anions


Storage of TCA Extract
Concentration (μM)
2000
1500
1000
70
60
50
40
30
20
10
0
0 hrs
24 hrs
36 hrs
Glycolate
Lactate
Pyruvate
P-glycolate
Anions


Storage of Neutralized TCA Extract
Concentration (μM)
2000
1500
1000
70
60
50
40
30
20
10
0
0 hrs
24 hrs
36 hrs
Glycolate
Lactate
Pyruvate
P-glycolate
Anions

RBC anions. Table 2 displays the anion concentrations for erythrocytes obtained from 30 normal human subjects compared with values previously published. Previously published values were unavailable for α-ketoglutarate, fructose-1,6-bisphosphate, fumarate, D-glycerate, glyoxylate, mannose-6-phosphate, and oxalate. Anion levels were similar to previously published values for ADP, ATP, 2,3-diphosphoglycerate, galactose-1-phosphate, glucose-6-phosphate, lactate, NADP, NADPH, phosphoenolpyruvate, 2- & 3-phosphoglycerate, phosphoglycolate, pyruvate, and ribose-5-phosphate. Anion levels were not similar to previously published values for fructose-6-phosphate, glycolate, IMP, and 6-phospogluconate.

RBC incubation experiments: glyoxylate. In an effort to determine the origin of oxalate in RBC, erythrocytes were incubated with glyoxylate, a known precursor of oxalate in the liver. A glyoxylate incubation concentration gradient was chosen based on the previously detected levels of glyoxylate in erythrocytes (see Table 2). RBC were incubated with varying concentrations of glyoxylate, beginning with the typical level (5μM) with a maximum of 1mM. Figure 10 displays the results of 10μM-1mM glyoxylate incubations. Figure 10(A) shows that as the concentration of glyoxylate increases as the concentration of intracellular glycolate increases. This suggests that the glyoxylate is entering the RBC and is being converted to glycolate, presumably by GR/HPR. Additionally, as the concentration of glyoxylate increases, the concentration of oxalate also increases. This is presumed to be due to the enzymatic activity of lactate dehydrogenase (LDH) which can convert glyoxylate to oxalate. No significant change in phosphoglycolate concentration was observed. Figure 10(B) shows that the concentrations of DPG and ATP, indicators of cellular energy levels, remain relatively stable during the glyoxylate incubations. This indicates that cellular

energy requirements were still being met, even at the rather unnatural 1mM glyoxylate concentration.

In order to investigate the effect of minor glyoxylate concentration changes, RBC were incubated with glyoxylate at levels in the lower concentration range (5μM, 10μM, 20μM, 35μM, 50μM), but no significant trends in anionic changes were observed.

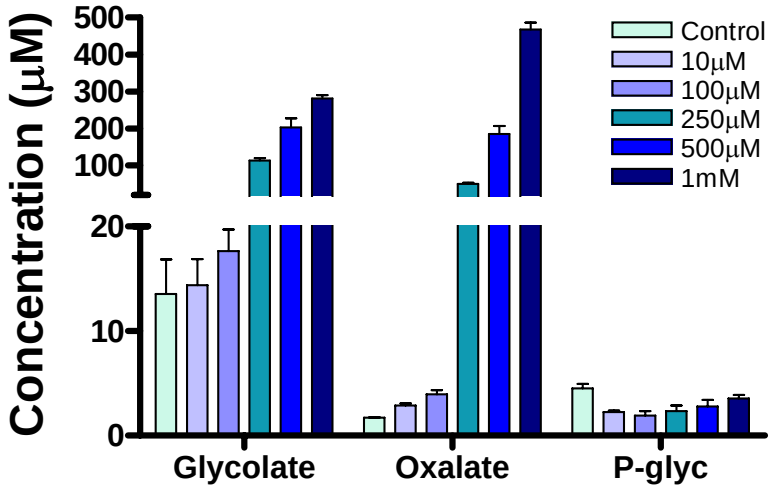
Table 2. Anions Levels Detected in Normal Human Erythrocytes.

Metabolite	**Recorded Values**		**Prior Published Values**	**Source**
	Mean*	SD	Range of Means*	
α-ketoglutarate**	1.70	0.562		
ADP	213.6	111.6	142 - 330	27,28,29,and 30
ATP	934.9	386.1	1100 - 1900	27,28,29,30,and 31
2,3-Diphosphoglycerate	1642.95	328.01	1670 - 7450	30,31,32,33,and 34
Fructose-6-PO_4	221	115	9.30 - 49.6	29,30,and 35
Fructose-1,6-bisPO_4**	62.28	41.94		
Fumarate**	6.28	3.88		
Galactose-1-PO_4	4.38	1.10	1.90	36
Glucose-6-PO_4	28.3	11.4	27.8 - 60.0	29 and 30
D-glycerate**	3.78	1.71		
Glycolate	8.11	2.56	70.4	7
Glyoxylate**	4.24	1.58		
IMP	24.64	9.53	8.10	30
Lactate	1160	411	932 - 1300	29,30,and 37
Mannose-6-PO_4**	21.9	16.7		
NADP	22.85	8.91	.065 - 38.0	27 and 30
NADPH	15.45	5.00	22.0 - 65.0	27 and 30
Oxalate**	2.92	1.06		
Phosphoenolpyruvate	15.5	8.99	8.10 - 12.2	29 and 30
6-phosphogluconate	1.77	0.510	45.0	30
2- & 3-phosphoglycerate	74.5	39.8	52.2 - 62.0	29 and 30
Phosphoglycolate	4.08	1.99	2.50 - 4.00	19, 38, and 39
Pyruvate	44.9	76.5	52.0 - 77.0	29, 30, 37
Ribose-5-PO_4	7.97	3.59	5.80	30

* Values expressed μmol/L packed red blood cells ** Previously undetected in human erythrocytes

Anion levels were determined using ion chromatography coupled with single quadrapole mass spectrometry. 30 normal subjects were sampled for each compound. For 24 subjects, n=2. For six subjects, n=3 due to re-sampling one year later. Mean anion levels were calculated as μmol/L packed red blood cells. Some prior published values not in these units were converted to μmol/L packed red blood cells.

(A)



(B)

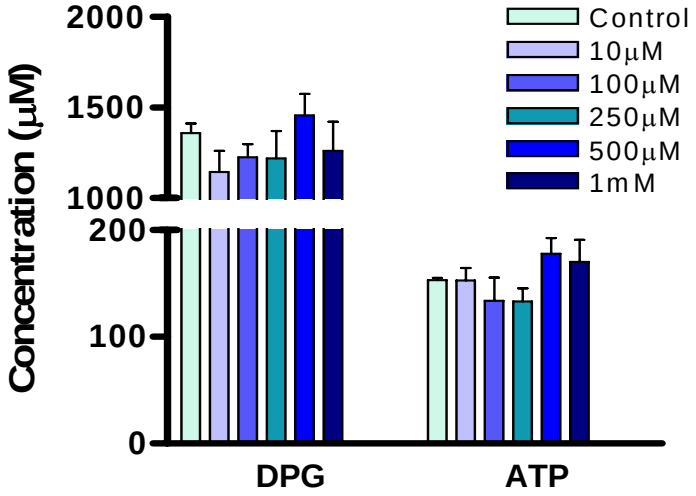
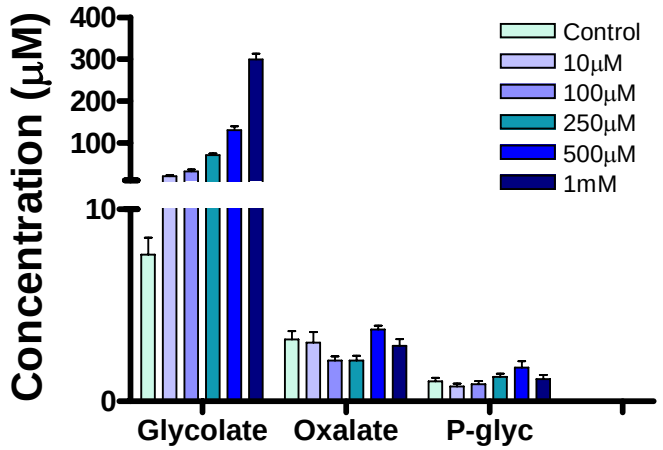


Figure 10. RBC Glyoxylate Incubations. Red blood cells were collected into pre-chilled, BD Vacutainer™ Sodium Heparin tubes. Blood was placed on ice immediately after blood draw and red cells were separated by centrifugation at 1,500 rcf for 5 min at 4ºC. Plasma and buffy coat were suctioned and red cells were washed three times with 1X PBS, pH 7.4, by vortexing, centrifuging at 1,500 rcf for 5 min at 4ºC, and suctioning the supernatant after each spin. After final wash and removal of supernatant, 200µl of packed red cells was transferred into a 24-well plate by using a positive-displacement pipette for precision. 800µl of Hank's Buffered Saline Solution containing glyoxylate at concentrations of 10µM, 100µM, 250µM, 500µM, 1mM was added to each well. For each concentration, 3 samples were incubated (n=3). Sample plates wrapped in foil (to protect from light and deter NADPH catabolism) were incubated for 1 hour at 37ºC on a shaking incubator to encourage oxygenation. (A) RBC Glyoxylate Incubation Results for Glycolate, Oxalate, and P-glycolate. Glycolate and Oxalate levels increased as glyoxylate concentrations increased. There was no significant trend in phosphoglycolate levels. (B) RBC Glyoxylate Incubation Results for DPG and ATP. There was no trend in DPG or ATP concentrations as glyoxylate concentrations increased.

RBC incubation experiments: glycolate. When it was determined that erythrocytes incubated with glyoxylate showed elevated intracellular oxalate levels, a follow up study was performed to investigate the likelihood of glycolate to contribute to oxalate toxicity. A glycolate incubation concentration gradient was chosen based on the previously detected levels of glycolate in erythrocytes (see Table 2).

Figure 11 displays the results of erythrocyte incubations with varying concentrations of glycolate in the media(10μm-1mM). Figure 11(A) shows that as the concentration of glycolate increases in the media, the concentration of intracellular glycolate increases. This indicates that the glycolate is being transported into the RBC. Additionally, as the concentration of glycolate increases, there is no significant trend in the concentration of oxalate or phosphoglycolate which indicates that glyoxylate is the necessary precursor for oxalate production and not glycolate. Figure 11(B) shows that the concentrations of DPG and ATP remain relatively stable during the glycolate incubations. This indicates that cellular energy requirements were still being met, even at the unnatural 1mM glycolate concentration.

(A)



(B)

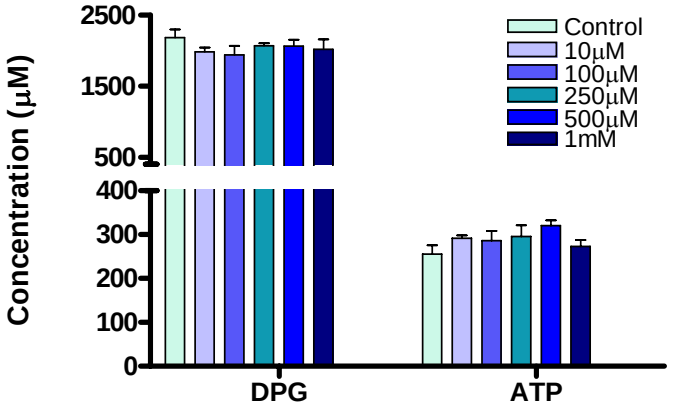
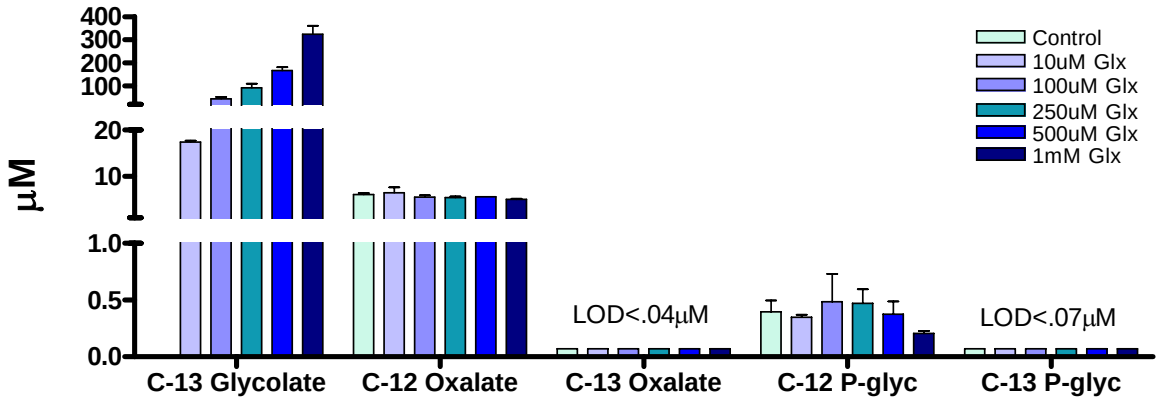


Figure 11. RBC Glycolate Incubations. Red blood cells were collected into pre-chilled, BD Vacutainer™ Sodium Heparin tubes. Blood was placed on ice immediately after blood draw and red cells were separated by centrifugation at 1,500 rcf for 5 min at 4ºC. Plasma and buffy coat were suctioned and red cells were washed three times with 1X PBS, pH 7.4, by vortexing, centrifuging at 1,500 rcf for 5 min at 4ºC, and suctioning the supernatant after each spin. After final wash and removal of supernatant, 200µl of packed red cells was transferred into a 24-well plate by using a positive-displacement pipette for precision. 800µl of Hank's Buffered Saline Solution containing glycolate at concentrations of 10µM, 100µM, 250µM, 500µM, 1mM was added to each well. For each concentration, 3 samples were incubated (n=3). Sample plates wrapped in foil (to protect from light and deter NADPH catabolism) were incubated for 1 hour at 37ºC on a shaking incubator to encourage oxygenation. (A) RBC Glycolate Incubation Results for Glycolate, Oxalate, and P-glycolate. Intracellular glycolate levels increased as glycolate concentrations increased. There was no significant trend in oxalate and phosphoglycolate levels. (B) RBC Glycolate Incubation Results for DPG and ATP. There was no trend in DPG or ATP concentrations as glycolate concentrations increased.

RBC incubation experiments: C-13 glycolate. RBC were incubated with C-13 glycolate to investigate the fate of glycolate as it is transported into RBC. Figure 12 displays the results of incubations with varying concentrations of C-13 glycolate (10μM-1mM). Figure 12(A) shows that as the concentration of C-13 glycolate increases the concentration of intracellular C-13 glycolate increases. This indicates that the C-13 glycolate is being transported into the RBC. No C-13 glycolate was detected in the control incubation. As the concentration of C-13 glycolate increased, neither C-13 oxalate nor C-13 phosphoglycolate was detected at levels greater than the limit of detection (LOD). This confirms that glycolate is not being converted to phosphoglycolate or contributing to oxalate production. Additionally, there was no significant trend in the concentration of C-12 oxalate or C-12 phosphoglycolate. Figure 12(B) shows that the concentrations of DPG and ATP remain relatively stable during the C-13 glycolate incubations. This indicates that cellular energy requirements were still being met, even at the rather unnatural 1mM C-13 glycolate concentration.

(A)



(B)

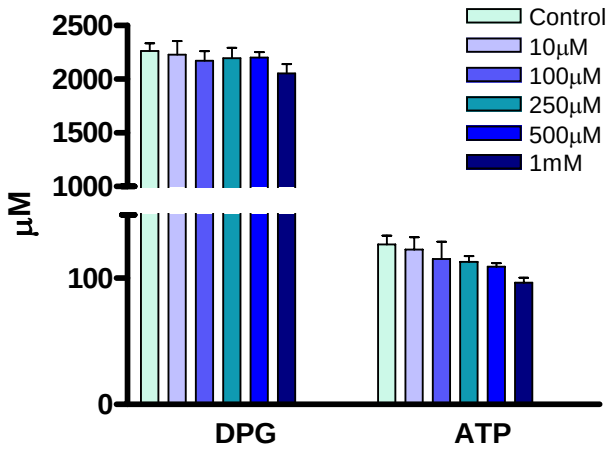


Figure 12. RBC C-13 Glycolate Incubations. Red blood cells were collected into pre-chilled, BD Vacutainer™ Sodium Heparin tubes. Blood was placed on ice immediately after blood draw and red cells were separated by centrifugation at 1,500 rcf for 5 min at 4ºC. Plasma and buffy coat were suctioned and red cells were washed three times with 1X PBS, pH 7.4, by vortexing, centrifuging at 1,500 rcf for 5 min at 4ºC, and suctioning the supernatant after each spin. After final wash and removal of supernatant, 200µl of packed red cells was transferred into a 24-well plate by using a positive-displacement pipette for precision. 800µl of Hank's Buffered Saline Solution containing C-13 glycolate at concentrations of 10µM, 100µM, 250µM, 500µM, 1mM was added to each well. For each concentration, 3 samples were incubated (n=3). Sample plates wrapped in foil (to protect from light and deter NADPH catabolism) were incubated for 1 hour at 37ºC on a shaking incubator to encourage oxygenation. (A) RBC C-13 Glycolate Incubation Results for C-13 Glycolate, C-12 Oxalate, C-13 Oxalate, C-12 Phosphoglycolate, and C-13 Phosphoglycolate. Intracellular C-13 glycolate levels increased as media C-13 glycolate concentrations increased. There was no significant trend in C-12 oxalate and C-12 phosphoglycolate levels. C-13 oxalate and C-13 phosphoglycolate were not detected at measurable levels. (B) RBC C-13 Glycolate Incubation Results for DPG and ATP. There was no trend in DPG concentrations as C-13 glycolate concentrations increased. There was a slight decreasing trend in ATP levels as C-13 glycolate increased.

Filter paper assay for DGDH activity detection in erythrocytes. Developing a standard protocol to diagnose PH2 patients quickly and cheaply has been a priority for researchers studying the hyperoxalurias for decades. Currently, the standard procedure to diagnose the disease is a series of genetic tests followed by a liver biopsy to confirm the presence or absence of enzymatic activity. This study investigated an assay that utilized Protein Saver filter paper to store whole blood samples at room temperature in a sealed envelope for up to 40 days. Table 3 reveals the results of those experiments.

Table 3. DGDH Activity in Human Dried Blood Spot (DBS)

Length of Storage	DGDH Activity
0 days	0.133 units
10 days	0.145 units
40 days	0.162 units

Whole blood from a finger prick was dropped directly onto Whatman ProteinSaver 903® filter paper. After drying blood spots for 4 hours at RT, 16mm pre-printed circles were cut from the membrane, sliced, and placed into a 1.7ml Eppendorf tube. A blood spot of 16mm absorbed approximately 50µl blood. Blood was extracted from filter paper by adding 1:10 solution of lysis buffer (450µl 0.1% Titon X-100) and incubating on ice for 10 min. Blood samples were then vortexed for an additional 10 min at RT. Lysates were then analyzed for DGDH activity. DGDH activity was measured by detecting hydroxypyruvate production by HPLC following a 20 minute assay and 15 minute phenylhydrazine (PH) incubation. In PH incubations, ketone bodies are derivatized by PH which has an absorbance of 313nm. This enables the detection of PH-derivatized analytes via UV spectrometry. The DGDH assay consisted of 20mM Tris (pH 8.5), 20mM D-Glycerate, and 1mM NADP. The DGDH assay was terminated by incubating with 1M perchloric acid after 20 minutes in a 37°C water bath. Centrifugation was used to remove protein precipitates and a 1mM phenylhydrazine 15 minute incubation was used to label α-keto acids. DGDH activity measurements were made for 6 samples at 0, 10, and 40 days in an envelope at room temperature. (1 unit activity = 1 nmol product/mg Hgb*min). Over 40 days, DBS retained DGDH activity.

DISCUSSION

The present study was designed to confirm the presence and investigate the functional role of GR/HPR in human erythrocytes and use a novel anion detection method that is highly accurate and sensitive to study oxalate metabolism. The study of oxalate metabolism is an important part of research surrounding stone-forming diseases as there is still much to be discovered about the metabolic precursor of oxalate and the proteins associated with its metabolism.

This study illustrates the use of ion chromatography coupled with mass spectrometry to detect anions that previously eluded scientists due to their co-elution using ion chromatography alone. This highly accurate and precise tool will enable scientists to conduct a host of future metabolic studies that were once considered futile due to difficulty surrounding anion detection. Ion chromatography coupled with mass spectrometry may be used to detect carboxylic acids, phosphosugars, and anions with sulphate groups.

In previous studies, phosphoglycolate was identified as a component of human erythrocytes, although its presence has been disputed by other scientists.[19] The current study shows the presence of phosphoglycolate as well as the detection of other intracellular anions at levels consistent with prior data including ADP, ATP, 2,3-DPG, glucose-6-phosphate, lactate, NADP, NADPH, phosphoenolpyruvate, 2- & 3-phosphoglycerate, pyruvate, and ribose-5-phosphate. However, this study detected some levels of anions that were inconsistent with previously recorded values. These anions were fructose-6-phosphate, galactose-1-phosphate, glycolate, IMP, and 6-phosphogluconate. These discrepancies can be attributed to the prior lack of availability for highly accurate and precise methods of detection, such as in this study. For example, in the case of the previously published fructose-6-phosphate levels, a method published by Beutler in 1986

was used[40]. This method involved diluting the sample, running it through a Dowex column, and then measuring the samples' phosphorus content via a very complicated procedure that entails aliquotting the Dowex eluent into centrifuge tubes, adding sulfuric acid and placing the samples in a 150ºC -160 ºC oven for 3 hours, cooling the samples and adding ammonium molybdate and the vortexing. Next, a reagent is added to neutralize the samples in a 100ºC water bath for 10 minutes. Following this process, the samples are then analyzed for UV measurement at 830nm. This previous method contains multiple opportunities for the loss of precision and accuracy and this may be why the levels we detected were higher.

In addition, seven previously-undetected anions were identified in human erythrocytes and normal ranges of concentrations were calculated using data from two blood draws from 30 subjects. Further confidence in the highly accurate method of measuring anions using ion chromatography coupled with mass spectrometry is evident by our re-sampling of six subjects one year after the initial measurements were taken and finding similar anion levels as previously detected. The coupling of the ion and mass separating techniques enables researchers to isolate anions that co-elute. The end result was a method of detecting 21 anionic acids in a one-pass method that is more sensitive and accurate than previous analytical methods. The development of this method could prove to be a useful tool in the investigation of erythrocyte metabolism.

Metabolites associated with oxalate metabolism were detected in human erythrocytes including an immediate metabolic precursor of oxalate, glyoxylate. The source of glyoxylate in erythrocytes, however, is yet to be determined. Glyoxal is currently being investigated by the Holmes laboratory as a potential source of glyoxylate in erythrocytes. Glyoxal is a dicarbonyl metabolic intermediate that can be synthesized in three different ways: the spontaneous degradation of glucose, the degradation of glycated proteins, and

lipid peroxidation.[41] High levels of glyoxal have been shown to be toxic to cells, therefore cells must utilize a system of detoxifying α-oxoaldehydes such as glyoxal. GR/HPR may assist in this detoxification as glyoxal can be oxidized to glyoxylate.

GR/HPR has long been known to be directly involved in oxalate metabolism; however, it has not been previously detected in erythrocytes. We have confirmed the presence of GR/HPR in human erythrocytes as well as its enzymatic activity at similar levels as leukocytes and BMC. Our initial observation of this activity is what prompted the present study. After confirming the presence and activity of GR/HPR, we further investigated its functional role in erythrocytes. It was initially hypothesized that the glyoxylate reductase activity of GR/HPR that converts glyoxylate to glycolate may provide the glycolate which can be phosphorylated to phosphoglycolate via pyruvate kinase. Phosphoglycolate is a potent activator of DPGM's phosphatase activity which hydrolyzes 2,3-DPG to 2-phosphoglycerate (PG) and a phosphate, thus decreasing the amount of free 2,3-DPG available to bind and stabilize deoxygenated hemoglobin which ultimately leads to hemoglobin's uptake of oxygen. We investigated GR/HPR's role in erythrocyte metabolism through a series of erythrocyte incubation experiments.

In the first set of erythrocyte incubation experiments, red cells were incubated with glyoxylate, a known metabolic substrate of GR/HPR, at varying concentrations beginning with levels similar to its intracellular concentration. In all glyoxylate experiments, glyoxylate was taken up by the red cells and corresponding increases in the levels of glycolate were observed. This result indicates that the cell membrane of the erythrocytes was able to transport glyoxylate into the cell and that GR/HPR acted on that glyoxylate by converting it to glycolate. Although, in these sets of experiments, a corresponding flux in phosphoglycolate was not observed. This finding indicates that the functional role of

GR/HPR in human erythrocytes may not be to provide glycolate for the production of phosphoglycolate. However, a closer look at the methods in these experiments may also shed light on the reason an increase in phosphoglycolate was not observed.

In typical erythrocyte incubations, red cells are purified from whole blood and dispensed into the wells of a 24-well plate along with an isotonic media and the analyte to be incubated. The plate would then be placed on a shaker in an incubator set at 37°C. While this method may suit its experimental purpose when studying aerobic glycolysis or other aerobic metabolic pathways, it may not be suitable for studying pathways that are initiated when red cells are under hypoxic conditions. One could hypothesize that in order for a flux in phosphoglycolate to be seen, the conditions that initiate the synthesis of phosphoglycolate must first be satisfied. In erythrocytes, the binding of 2,3-DPG to hemoglobin is crucial in maintaining proper oxygenation of tissues. 2,3-DPG is responsible for stabilizing deoxygenated hemoglobin thus decreasing its affinity for oxygen. Intracellular levels of 2,3-DPG are tightly regulated via a tri-functional enzyme known as diphosphoglycerate mutase (DPGM). The phosphatase activity of DPGM, is responsible for hydrolyzing 2,3-DPG to 2-phosphoglycerate (PG) and a phosphate. A potent activator of this phosphatase activity is phosphoglycolate. An increase in phosphoglycolate levels would result in a net decrease in 2,3-DPG levels would be hypoxic conditions where the red cells would need to maintain stable oxygen levels in tissues. In typical erythrocyte incubations, the red cells are incubated in hyperoxygenated conditions. In order to definitively prove that the function of GR/HPR is not to provide glycolate for the synthesis of phosphoglycolate in erythrocytes, red cell incubations need to be conducted in hypoxic conditions.

Another indicator that the erythrocytes in our incubation experiments were not enduring hypoxic stress would be the maintenance of cellular energy levels. In all of our experiments, levels of 2,3-DPG and ATP remained stable. This was a particularly surprising discovery given the overwhelmingly high levels of glyoxylate (up to 1mM) with which we were incubating the red cells. At these extremely high levels of glyoxylate, one would assume that the cells metabolic machinery would become overwhelmed and show signs of stress. These results are a prime example of our gross underestimation of the ability of red cells to survive in seemingly extreme conditions. One could argue that perhaps the cells were not transporting the glyoxylate into the cell, but the levels of intracellular glycolate fluxes that were detected (up to 300μM) clearly indicate the contrary. One could also argue that our one hour incubation time did not allow enough time for cells to undergo stress, but when pilot experiments were conducted at the beginning of the study, red cells were incubated at upwards of 4 hours, at which time they only showed slight increases in 2,3-DPG.

Another important result of the glyoxylate experiments to note is the synthesis of oxalate when red cells are incubated with glyoxylate, presumably by lactate dehydrogenase (LDH) acting on glyoxylate. Further, erythrocytes show a dose response - as glyoxylate incubation levels increased, intracellular oxalate levels also increased. This is of importance for two reasons: firstly, oxalate has not been previously detected in erythrocytes, thus our experimentation proved that it was not only detectable, but we could also recreate the conditions under which it is synthesized; secondly, is that the generation of oxalate in erythrocytes as a response to the glyoxylate increase sheds light on a previously unknown metabolic pathway. It has now been proven that oxalate, a major component of 80% of kidney stones formed in patients, can be synthesized in human erythrocytes and the

conditions surrounding that synthesis has been mimicked *in vitro*. This important discovery of a previously unconfirmed metabolic pathway in erythrocytes, could lead to better-targeted treatment of not only Primary Hyperoxaluria Type 2, but also other stone-forming diseases that result from the formation of calcium oxalate stones.

In the second set of erythrocyte incubation experiments, red cells were incubated with glycolate, GR/HPR's known metabolic product, at varying concentrations beginning with levels similar to its intracellular concentration. These experiments were conducted in an effort to prove that glyoxylate was in fact the metabolic precursor of oxalate in erythrocytes. Additionally, we wanted to investigate whether or not glycolate would be taken up by erythrocytes more readily than glyoxylate. In all experiments, glycolate was taken up by the red cells and corresponding increases in the levels of intracellular glycolate were observed. This indicates that the cell membrane of the erythrocytes was able to transport glycolate into the cell. Although in these experiments, a corresponding flux of oxalate was not observed. This finding indicates that glycolate is not the metabolic precursor of oxalate in human erythrocytes and further confirms our previous finding that glyoxylate is a precursor of oxalate. In addition, no increase in intracellular phosphoglycolate levels was observed. From this second set of experiments, it can be concluded that glycolate is transported into erythrocytes, glycolate is not a metabolic precursor of oxalate, and that the conditions of these incubations were not favorable for the synthesis of phosphoglycolate. At the end of the glyoxylate and glycolate experiments, the metabolic fate of the glycolate flux that occurs after incubation with both analytes remains to be seen.

The next set of experiments were incubations using C-13 glycolate to investigate the metabolic fate of a glycolate flux. All aspects of the previous erythrocyte incubations

remained constant, with the exception of the use of a glycolate isotope, C-13 glycolate. This isotope is present on every carbon atom, so regardless of the fate of the C-13 glycolate, we should be able to discern where it went by collecting the correct SIM on the IC-MS apparatus by adding one mass unit to each of the 21 analytes we are able to measure. The same concentrations of C-13 glycolate were used as in the previous glycolate experiments. After the one hour incubation, the levels of both the C-12 analytes and C-13 analytes were measured. In all instances, a flux of C-13 glycolate was observed, but levels of C-12 analytes remained constant, including C-12 glycolate, C-12 oxalate, and C-12 phosphoglycolate. Further, there were no detectable C-13 analytes in any of the incubations, with the exception of C-13 glycolate. The C-13 glycolate experiments did not reveal the destination of glycolate in red cells.

Although the fate of glycolate was undetermined in these experiments, it is important to note the potential metabolic contribution that erythrocyte GR/HPR is capable of making. Composed of an average of 20-30 trillion cells in the body and making up 7% of a human's body weight, the biomass of erythrocytes is substantial. A flux in glyoxylate concentration of a seemingly miniscule amount, 2μM for example, can amount to an appreciable amount when considering the 2.5 liters of erythrocytes that are contained in the human body. Further, now that oxalate metabolism has been confirmed in human erythrocytes, scientists can begin to consider erythrocytes as a contributor when devising future investigations of oxalate metabolism.

Although GR/HPR's role in erythrocytes was not entirely worked out in these studies, it is important to note that even at 1mM concentration of incubated glycolate or glyoxylate, cellular energy levels remained stable - including NADPH. This indicates that GR/HPR might play a role in the maintenance of intracellular energy levels, because

NADPH is its co-factor. GR/HPR could be responsible for both removing glyoxylate, a major precursor of oxalate, and maintaining stable NADPH levels.

In addition to discovering the potential contribution GR/HPR makes in the metabolism of oxalate in erythrocytes, we also investigated the utilization of this discovery for the development of a less-invasive diagnostic assay for the detection of Primary Hyperoxaluria Type 2. The Dried Blood Spot Filter Paper Assay has been used to diagnose metabolic and protein disorders since 1964. They are used in over 20 countries in the early detection of diseases such as phenylketouria, congenital hypothyroidism, sickle cell and HIV. This assay could prove to be a more time-efficient and cost-effective solution to early detection of Primary Hyperoxaluria Type 2. By requiring merely two drops of blood, the tissue it utilizes is much more readily available than a liver biopsy and the procedure to obtain the tissue is less invasive to the patient. Additionally, the Dried Blood Spot Filter Paper Assay does not require the expensive shipping of ice-cold samples as in the liver-biopsy assay. In fact, samples remained stable for up to 40 days at room temperature in an envelope. However, in order to definitively prove this, further studies that include analysis of PH2 patient blood would be necessary.

In conclusion, this study shed light on a previously un-described metabolic pathway in human erythrocytes. Oxalate metabolism was found to exist in human erythrocytes, a phenomenon previously thought to occur exclusively in the liver and kidneys. Further investigation of this pathway could potentially lead to a greater understanding of oxalate metabolism, better diagnostic assays, and superior treatment strategies for Primary Hyperoxaluria Type 2.

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