Quantification of Oxalate in Human Plasma by Novel Liquid Chromatography–Tandem Mass Spectrometry: Method Development, Validation, and Application in Lumasiran Clinical Trials

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Conclusions

- A novel LC-MS/MS assay was developed and validated successfully and in accordance with regulatory guidelines
- The ULN was calculated as 12.11 µmol/L with the normal range of 1.71–12.11 µmol/L from 75 healthy adult volunteers
- The required sample volume was only 100 µL of K_2EDTA plasma, which is especially favorable in the pediatric population, and there is no need to acidify blood samples before processing
- The assay accurately determines POx levels, which were used as an efficacy endpoint in the clinical development of lumasiran

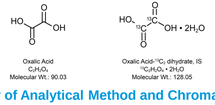
Introduction

- Primary hyperoxaluria type 1 (PH1) is a rare, autosomal recessive genetic disease characterized by hepatic oxalate overproduction[1,2]
- Oxalate is excreted primarily by the kidneys; urinary oxalate can combine with calcium and cause kidney stones and/or nephrocalcinosis, leading to progressive kidney damage[3]
- As kidney function declines, POx increases. POx should be monitored in PH1 patients with compromised kidney function,[3-5] but measuring POx is challenging due to its intrinsic chemical property and nonenzymatic conversion of ascorbate to oxalate in vitro[6-8]
- A number of methods to measure POx with differing procedures for sample preparation have been developed with varying normal ranges; methods include enzymatic oxalate oxidase,[6,9] gas chromatography,[6] HPLC,[10] IC,[11] and LC-MS/MS[12]
- We present the methodology used for development and validation of a novel LC-MS/MS assay to determine oxalate concentration in human K_2EDTA plasma

Methods

- A validated LC-MS/MS assay capable of measuring oxalate in 100 µL of K_2EDTA plasma was developed
- Samples were frozen on dry ice and stored in a −70 °C freezer
- Samples were spiked with internal standard ($^{13}C_2$-labeled oxalic acid; **Figure 1**), acidified, and extracted by protein precipitation prior to analysis using anion exchange HPLC with electrospray ionization MS/MS detection
- Analytical method and chromatography settings are summarized in **Table 1**

Figure 1. Chemical Structures of Analyte and Internal Standard



Oxalic Acid
$C_2H_2O_4$
Molecular Wt.: 90.03

Oxalic Acid-$^{13}C_2$ dihydrate, IS
$^{13}C_2H_2O_4 \cdot 2H_2O$
Molecular Wt.: 128.05

Table 1. Summary of Analytical Method and Chromatography Settings

General	
Analyte name	Oxalate
Internal standard	$^{13}C_2$-labeled oxalic acid
Matrix	K_2EDTA human plasma
Calibration standard concentrations	0.500, 0.100, 2.50, 5.00, 10.0, 20.0, 45.0, and 50.0 µg/mL
Quality control concentrations	0.500 in surrogate matrix (3% BSA in water solution); endogenous, endogenous + 4.00, endogenous + 16.0, and endogenous + 40.0 µg/mL in authentic matrix (K_2EDTA human plasma)
Regression type	Linear analysis with $1/x^2$ weighting using Watson LIMS version 7.4.1
Sample volume	100 µL
Chromatography Settings	
Mobile phase	A: acetonitrile/water/1 M ammonium bicarbonate/acetic acid at 20:80:1:0.05 [v/v/v/v] B: acetonitrile/water/1 M ammonium bicarbonate at 20:70:10 [v/v/v]
Flow rate	800 µL/min
Run time	4 min
Injection volume	10 µL
Mass Spectrometer Settings	
Mass spectrometer	SCIEX 6500 mass spectrometer
Ionization mode	Turbo Ion Spray set in a negative ionization mode using multiple reaction monitoring
Ion spray voltage	−4500 V
Scan duration	2 min
Dwell time	0.1 sec
Collision energy	
Oxalate	−12 eV
Oxalate-$^{13}C_2$	−12 eV
m/z transitions	
Oxalate	89.0→61.0
Oxalate-$^{13}C_2$	91.0→62.0

Validation

- The method was assessed for linearity, sensitivity, accuracy, precision, selectivity, hemolyzed plasma, lipemic plasma, interference, parallelism, recovery, matrix effect, and stability

Application to Samples from the Lumasiran Clinical Studies

- The validated LC-MS/MS assay was used to quantify POx in the clinical development of lumasiran (**Figure 2**)

Figure 2. Lumasiran Clinical Studies

Phase 1/2 and Phase 2 OLE

Phase 1/2
NCT02706686

Phase 2 OLE
NCT03350451

- Plasma samples were tested using the clinical IC method, and later, additional plasma samples were tested using the validated LC-MS/MS method

ILLUMINATE-A, ILLUMINATE-B, and ILLUMINATE-C

ILLUMINATE-A
NCT03681184

ILLUMINATE-B
NCT03905694

ILLUMINATE-C
NCT04152200

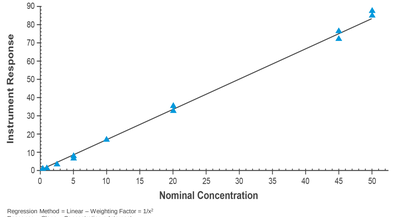
- The validated LC-MS/MS assay was used as the primary method for POx quantification
- The IC assay continued to be used in parallel with the LC-MS/MS assay to allow for comparison of results with historical and published data. In ILLUMINATE-A, samples for IC analysis were collected through Month 6. In ILLUMINATE-B, only baseline samples for IC analysis were collected due to blood volume limitations

Results

Linearity and Sensitivity

- The LLOQ for this assay was 0.500 µg/mL for oxalate
- The back-calculated calibration standard concentrations for oxalate in surrogate matrix (3% BSA) showed a linear fit from 0.500 to 50.0 µg/mL
- An example of a standard curve regression for oxalate is shown (**Figure 3**)

Figure 3. Standard Curve Regression for Oxalate from Run 1



Regression Method = Linear – Weighting Factor = $1/x^2$
Response = Slope × Concentration + Intercept
Slope = 1.66546512; Intercept = 0.109148317; R^2 = 0.9963

Accuracy and Precision

- Results from the intra-run and inter-run accuracy and precision evaluations were acceptable (**Table 2**)

Selectivity

- The accuracy and precision data for 4.00 µg/mL of oxalate in the 6 individual lots of human plasma were within the acceptable range
- Accuracy (%RE) of the assay ranged from 0.5 to 9.8

Hemolyzed Plasma Test and Lipemic Plasma Test

- The results of both the hemolyzed plasma test and the lipemic plasma test met the acceptance criteria (%CV ≤15)

Interference Test

- The interference test met the acceptance criteria (%RE=14.1 and 5.5, respectively)

Parallelism Test

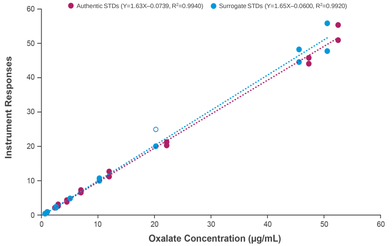
- The back-calculated concentrations of oxalate calibration standards in surrogate matrix and authentic matrix and regression parameters were acceptable (**Figure 4**)

Table 2. Intra-run and Inter-run Accuracy and Precision of Oxalate in Surrogate Matrix (3% BSA in Water Solution) and Authentic Matrix (K_2EDTA Human Plasma)

Curve No.	LLOQ QC (Water) 0.500 µg/mL	LQC-PL Endogenous, 1.80 µg/mL[a]	LMQC-PL Endogenous + 4.00 µg/mL = 5.80 µg/mL[a]	HMQC-PL Endogenous + 16.0 µg/mL = 17.8 µg/mL[a]	HQC-PL Endogenous + 40.0 µg/mL = 41.8 µg/mL[a]
1	0.494	1.73	5.80	18.3	42.5
	0.524	1.79	5.94	18.8	42.4
	0.556	1.48	5.80	18.5	42.9
	0.548	1.71	5.95	16.7	43.6
	0.439	1.72	5.95	18.8	43.9
	0.506	1.65	5.94	18.5	43.3
Intra-run mean	0.511	1.68	5.90	18.3	43.1
SD	0.0426	0.110	0.0750	0.792	0.603
%CV	8.3	6.4	1.3	4.3	1.4
%RE	2.2	0.0	3.9	3.4	3.4
N	6	6	6	6	6
2	0.587	2.10	6.16	18.0	42.5
	0.588	1.88	6.09	17.8	43.0
	0.565	2.12	5.79	17.4	43.4
	0.581	1.99	5.60	18.2	42.3
	0.559	1.92	6.07	17.7	41.9
	0.577	2.33	5.50	16.4	42.0
Intra-run mean	0.577	2.06	5.87	17.6	42.5
SD	0.0122	0.164	0.279	0.640	0.585
%CV	2.1	8.0	4.8	3.6	1.4
%RE	15.4	0.0	−3.1	−2.8	1.0
N	6	6	6	6	6
3	0.559	1.66	6.01	19.1	42.4
	0.477	1.66	5.96	18.2	41.4
	0.477	1.68	5.37	18.4	43.0
	0.471	1.71	5.46	18.4	42.7
	0.546	1.59	5.94	18.3	42.4
	0.510	1.69	5.90	18.4	41.9
Intra-run mean	0.507	1.67	5.77	18.5	42.3
SD	0.0383	0.0410	0.281	0.320	0.573
%CV	7.6	2.5	4.9	1.7	1.4
%RE	1.4	0.0	1.8	4.5	1.4
N	6	6	6	6	6
Inter-run mean	0.531	1.8	5.85	18.1	42.6
Inter-run SD	0.0455	0.216	0.225	0.697	0.652
Inter-run %CV	8.6	12.0	3.8	3.9	1.5
Inter-run %RE	6.2	0.0	0.9	1.7	1.9
N	18	18	18	18	18

[a] Mean of endogenous concentrations from three accuracy and precision runs was used as nominal endogenous value for statistical analysis.

Figure 4. Two Calibration Curves in Surrogate Matrix (3% BSA in Water Solution) and Authentic Matrix (K_2EDTA Human Plasma) for Parallelism Test (Run 4)



Dotted lines represent lines of best fit. White dot with blue outline (20.0, 24.8) was excluded from the surrogate STDs linear fit assessment.

Recovery and Matrix Effect

- The recovery of oxalate and the IS met the acceptance criteria and was sufficiently consistent across the range tested
- The matrix effect test met the acceptable criteria and had no significant impact on the study data

Stability

- The freeze-thaw, benchtop, processed sample, and whole blood stability all met the acceptance criteria
- Stability results are summarized in **Table 3**

Table 3. Summary of Stability

Freeze-thaw in K_2EDTA human plasma	5 cycles
Benchtop in K_2EDTA human plasma	24 h at ambient temperature
Processed sample	139 h at 4 ˚C
Whole blood	2 h at ambient temperature
Long-term storage in K_2EDTA human plasma	125 d at −70 ˚C 216 d at −20 ˚C

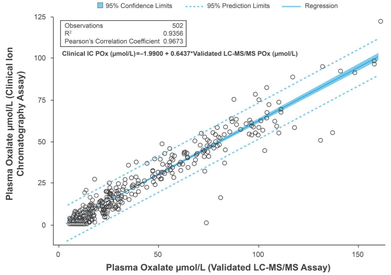
Reference Range Evaluation

- POx was assessed in 75 healthy adults to determine the normal range and ULN
- Among 75 healthy adults, the normal range was 1.71–12.11 µmol/L
- The 99th percentile of the Weibull distribution for POx was 12.11 µmol/L and is regarded as the ULN

Results from Samples from the Lumasiran Clinical Studies

- The POx values obtained using the LC-MS/MS assay were generally greater than the values using the IC method. However, the results were broadly correlated, with a Pearson correlation coefficient of 0.9673 (**Figure 5**)
- Regression equation: Clinical IC POx (µmol/L)= −1.9900 + 0.6437*Validated LC-MS/MS POx (µmol/L) (**Figure 5**)

Figure 5. Comparison Plot of POx Levels in Samples from Lumasiran Clinical Studies Quantified Using Clinical IC and Validated LC-MS/MS Methods[a]



[a] Lumasiran clinical studies include Study 002 (Phase 2 OLE), ILLUMINATE-A, ILLUMINATE-B, and ILLUMINATE-C.

Acknowledgments: Thank you to the patients, their families, investigators, study staff, and collaborators for their participation in the lumasiran clinical studies. Thank you to the team members at QPS, LLC, for their contribution in the development of this assay. Medical writing and editorial assistance was provided by Peloton Advantage, LLC, an OPEN Health company, in accordance with Good Publication Practice (GPP3) guidelines and funded by Alnylam Pharmaceuticals. **Funding:** This study was funded by Alnylam Pharmaceuticals.

Disclosures: VAC, JMG, GJR, and **J-TW:** employees of Alnylam Pharmaceuticals and are shareholders or hold stock options from Alnylam Pharmaceuticals. **KHC:** former employee of Alnylam Pharmaceuticals and is a shareholder or holds stock options from Alnylam Pharmaceuticals.

Abbreviations: %CV, coefficient of variation; %RE, percent relative error; BSA, bovine serum albumin; HMQC, high medium quality control; HPLC, high-performance liquid chromatography; HQC, high quality control; IC, ion chromatography; IS, internal standard; K_2EDTA, dipotassium ethylenediaminetetraacetic acid; LC-MS/MS, liquid chromatography–tandem mass spectrometry; LLOQ, lower limit of quantification; LMQC, low medium quality control; LQC, low quality control; OLE, open-label extension; POx, plasma oxalate; QC, quality control; SD, standard deviation; STD, standard; ULN, upper limit of normal.

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Presented at: ASN Kidney Week 2022; November 3–6, 2022; Orlando, FL and Virtual