A Robust, Simple, High-Throughput UPLC-MS/MS Assay To Quantify a Novel Biomarker, Phenylacetylglutamine, in Human Plasma

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Introduction

- In phenylketonuria, mutations of the phenylalanine hydroxylase (PAH) gene decrease the ability of PAH to convert phenylalanine to tyrosine, resulting in Phe accumulation in the blood and brain and disruption of neurotransmitter biosynthesis and metabolism. Phenylacetylglutamine (PhenylAc-Gln-OH or PAG) is the primary product from phenylacetic acid, which is one of the neurotransmitter metabolites (**Figure 1**). PAG was later identified as a novel biomarker in acute ischemic stroke.
- It is important to quantify the levels of the monoamine neurotransmitter metabolites accurately in human plasma in order to support clinical studies. To this end, we developed and validated a rapid, robust, high-throughput UPLC-MS/MS method to measure the concentration of PAG in human sodium heparin (NaHep) plasma

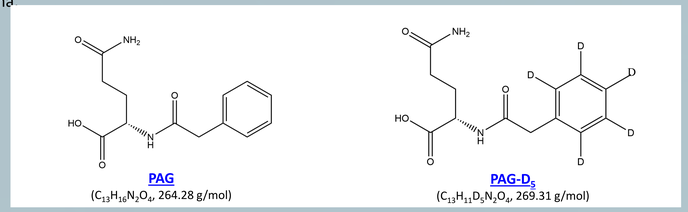

Figure 1: Chemical structures of phenylacetylglutamine (PAG) and its internal standard deuterated PAG (PAG-d_5)

Method Challenges

Neurotransmitter metabolite PAG is a novel biomarker in acute ischemic stroke. A robust, simple, high-throughput ultra-performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS) assay was developed and validated to quantify PAG in human plasma with high endogenous levels of PAG. The assay has the following attributes:

- **Compound stability and non-specific binding evaluation:** To understand the properties of PAG regarding the stability and non-specific binding, the stress tests for PAG in neat solution, surrogate matrix, and authentic matrix were conducted (**Figure 2** and **Figure 3**). All the tests confirmed the conditions for working solution, surrogate matrix, and provided the reference for the stability for validation.
- **Surrogate matrix selection for high endogenous level compounds:** Human plasma has high endogenous levels of PAG (screened at ~500 nM); thus, an appropriate surrogate matrix was required for this assay. Different types of potential surrogate matrices were compared and evaluated. The surrogate matrix that was selected was 5% bovine serum albumin (BSA) in water because it mimics the protein level in plasma without the endogenous issues, solubility issues, or stability issues of PAG. Good parallelism between the surrogate matrix and human plasma was obtained (**Table 1** and **Table 2**). A decent recovery rate was also achieved in both surrogate matrix and authentic matrix (**Table 3**).
- **Simplicity:** Only a one-step protein precipitation was used to extract the PAG from human plasma. The total extraction time was less than 15 minutes.
- **High throughput:** Different columns and mobile phases were compared and evaluated, and the HALO® 90 Å Biphenyl LC column (2 µm, 2.1 × 75 mm; Advanced Materials Technology, Wilmington, DE) was selected along with most common but robust mobile phases (mobile phase A: 0.1% formic acid in water; mobile phase B: 0.1% formic acid in acetonitrile). The total chromatography run time was 3 minutes per sample, and the total elapsed time to process one full 96-well plate was ~4.8 hours.

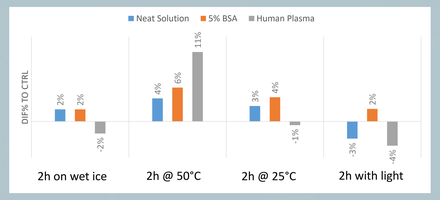

Figure 2: Stability of PAG in neat solution, 5% BSA, and human plasma

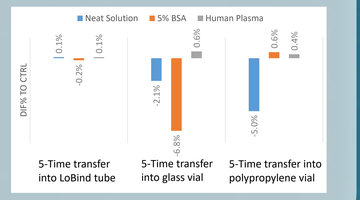

Figure 3: Non-specific binding of PAG in neat solution, 5% BSA, and human plasma

Method Challenges (*Cont.*)

Table 1: Dilution linearity of PAG in different surrogate matrices

Surrogate Matrix Type	Accuracy (%)							
	Std-1	Std-2	Std-3	Std-4	Std-5	Std-6	Std-7	Std-8
MeOH/H_2O (1:19, *v/v*)	85.2	92.2	98.2	94.2	104.5	106.2	104.2	106.2
5% BSA in H_2O	99.8	101	99.0	101	99.3	99.6	98.7	102.4

Table 2: Dilution parallelism of surrogate matrix 5% BSA

Dilution Parallelism	Concentration (nM) of PAG		
	15,512* (5x Dilution)	15,512* (10x Dilution)	15,512* (100x Dilution)
Accuracy (%):	106.4	101.9	93.5
*Dilution QCs with endogenous level = Average endogenous level (539 nM) x 95% + [Concentration of working solution (300,000 nM) x 5%]			

Table 3: Recoveries of PAG and internal standard PAG-D_5 from surrogate matrix (5% BSA) and authentic matrix (human plasma)

Matrix Type	Instrument Response			
	PAG		PAG-d_5	
	Extracted QC Sample	Recovery Sample Peak Area	IS-Extracted QC Sample	IS Recovery Sample Peak Area
5% BSA	43,214	45,868	134,017	145,772
	43,723	47,646	135,188	146,272
	41,064	48,176	140,794	149,669
n	3	3	3	3
Mean	42,667	47,230	136,666	147,238
SD	1411	1209	3622	2120
CV (%)	3.3	2.6	2.7	1.4
Recovery (%)	N/A	90.3	N/A	92.8
Human Plasma	1,021,337	1,032,931	145,100	149,648
	988,210	1,064,289	146,344	148,615
	1,009,891	1,036,634	143,612	149,860
n	3	3	3	3
Mean	1,006,479	1,044,618	145,019	149,374
SD	16,825	17,136	1368	666
CV (%)	1.7	1.6	0.9	0.4
Recovery (%)	N/A	96.3	N/A	97.1

Finalized Method

A 25.0-µL sample of human plasma (NaHep) was extracted with internal standard PAG-d_5 by protein precipitation using acetonitrile (ACN). The extraction supernatant was further diluted with 0.1% formic acid in water before analysis. Extracts were injected onto a HALO® 90 Å Biphenyl LC column (2 µm, 2.1 × 75 mm) at a column temperature of 40°C. The mobile phase consisted of a solvent mixture of formic acid, water, and ACN using a shallow gradient at 0.5 mL/min for 3 min. A SCIEX API (atmospheric pressure ionization) 5500 UPLC-MS/MS system was used in electrospray ionization (ESI) positive mode to monitor PAG and internal standard PAG-d_5 at ion transitions of 265.1→130.0 and 270.2→130.0, respectively.

Results

The UPLC-MS/MS assay was successfully developed and fully validated in human plasma within a quantification range of 50.0 to 5000 nM (**Figure 4**). The validation experiments included intra-day and inter-day precision and accuracy, sensitivity, selectivity/specificity, matrix effect, various stability tests, recovery, and dilution integrity. Typical chromatograms of the lower-limit-of-quantification quality control (LLOQ-QC), upper-limit-of- quantification QC (ULOQ-QC), matrix blank, and control-zero are shown in **Figure 5**. The inter-day accuracy (% bias) results for three runs of LLOQ-QC, LQC, MQC, and HQC were 94.4%, 96.0%, 100.7%, and 96.8%, respectively; the inter-day precision (%CV) results for three runs of LLOQ-QC, LQC, MQC, and HQC were 5.2%, 3.9%, 4.6%, and 3.5%, respectively (**Table 4**). Selectivity was successfully determined without observable interference using six different lots of matrix.

Sixteen (16) hours of benchtop matrix stability at room temperature, four (4) cycles of freeze-thaw stability, and 3 months (97 days) of long-term matrix stability at −70°C were successfully established. Seventy-seven (77) hours of processed sample stability was established at 2–8°C (**Table 5**). Dilution linearity was successfully evaluated for 5-times dilution. This validated method was successfully applied to a preclinical pharmacokinetic study.

Results (*Cont.*)

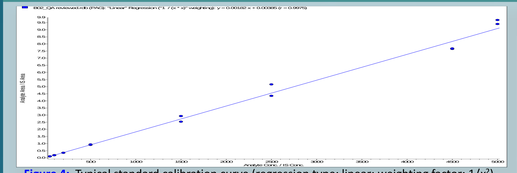

Figure 4: Typical standard calibration curve (regression type: linear; weighting factor: 1/x^2)

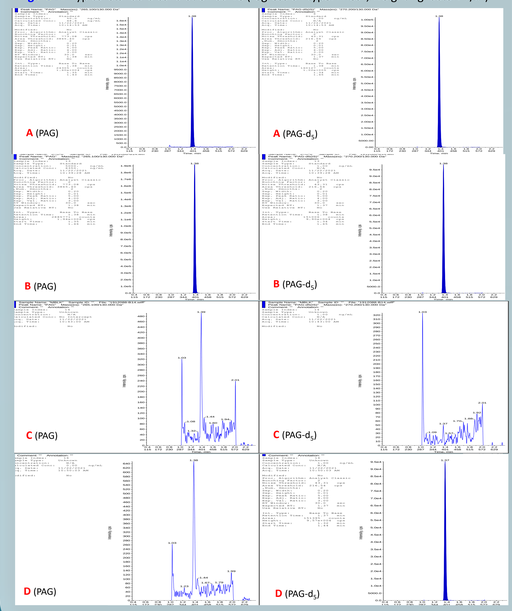

Figure 5: Typical chromatograms of LLOQ-QC (**A**), ULOQ-QC (**B**), matrix blank (**C**), and control-zero (**D**) samples.

Table 4: Inter- and intra-accuracy and precision

Batch ID	Concentration (ng/mL)							
	LLOQ-QC 50.0	DEV (%)	LQC 150	DEV (%)	MQC 1112 [a]	DEV (%)	HQC 3512 [b]	DEV (%)
02	48.5	-3.0	150	-0.1	1080	-2.9	3320	-5.5
	48.6	-2.8	148	-1.5	1080	-2.9	3270	-6.9
	48.4	-3.2	137	-8.6	1080	-2.9	3250	-7.5
	48.3	-3.4	142	-5.3	1100	-1.1	3260	-7.2
	48.2	-3.6	150	0.0	1090	-2.0	3310	-5.8
	46.8	-6.4	146	-2.9	1100	-1.1	3240	-7.7
n	6		6		6		6	
Mean	48.1		145		1090		3280	
SD	0.668		5.04		9.83		32.7	
CV (%)	1.4		3.5		0.9		1.0	
Accuracy (%)	96.2		96.7		98.0		93.4	
05	47.6	-4.8	155	3.3	1150	3.4	3510	-0.1
	43.7	-12.6	143	-4.7	1290	16.0	3350	-4.6
	42.7	-14.6	135	-10.0	1130	1.6	3280	-6.6
	48.5	-3.0	151	0.7	1150	3.4	3470	-1.2
	45.3	-9.4	143	-4.7	1130	1.6	3590	2.2
	41.8	-16.4	138	-8.0	1170	5.2	3520	0.2
n	6		6		6		6	
Mean	44.9		144		1170		3450	
SD	2.69		7.60		60.7		116	
CV (%)	6.0		5.3		5.2		3.4	
Accuracy (%)	89.8		96.0		105.2		98.7	
09	49.4	-1.2	212 [c]	N/A	1070	-3.8	3490	-0.6
	48.2	-3.6	139	-7.3	1120	0.7	3490	-0.6
	50.3	0.6	142	-5.3	1080	-2.9	3530	0.5
	49.7	-0.6	139	-7.3	1100	-1.1	3530	0.5
	47.1	-5.8	144	-4.0	1120	0.7	3420	-2.6
	32.4 [c]	N/A	148	-1.3	1110	-0.2	3290	-6.3
N	5		5		6		6	
Mean	48.9		142		1100		3460	
SD	1.28		3.78		21.0		91.7	
CV (%)	2.6		2.7		1.9		2.7	
Accuracy (%)	97.8		94.7		98.9		98.5	
Inter-assay:								
n	17		17		18		18	
Overall Mean	47.2		144		1120		3400	
SD	2.45		5.58		51.2		120	
CV (%)	5.2		3.9		4.6		3.5	
Accuracy (%)	94.4		96.0		100.7		96.8	

N/A: Not applicable

[a] MQCs with endogenous level = Average endogenous level (539 nM) x 95% + [Concentration of working solution (12,000 nM) x 5%]

[b] HQCs with endogenous level = Average endogenous level (539 nM) x 95% + [Concentration of working solution (60,000 nM) x 5%]

[c] Determined to be an outlier.

Table 5: Stability Data

Parameter	Established Stability
Long-term storage at – 70°C	97 days
Benchtop room temperature (RT) storage	16 hours
Processed sample stability at 4°C	77 hours
Freeze/thaw (– 20°C/room temp) stability	4 cycles

Conclusions

A robust, simple, high-throughput UPLC-MS/MS assay was successfully developed and validated to quantify a novel biomarker, phenylacetylglutamine, in human NaHep plasma within an assay range of 50.0 to 5000 nM. The assay has the following advantages:

- ***Suitable Surrogate Matrix Selection:*** Human plasma has relatively high endogenous levels of PAG. Optimized surrogate matrix was selected and proved to be reliable in recovery and parallelism.
- ***Simplicity:*** This robust but simple extraction method can be conducted by an entry-level trained analyst in less than 15 minutes.
- ***Precision and Accuracy:*** This assay was fully validated in accordance with U.S. Food and Drug Administration *Bioanalytical Method Guidance for Industry* (2018) and showed good precision and accuracy.
- ***Higher Throughput:*** The total elapsed time to process one full 96-well plate was ~4.8 hours. The total chromatography run time was 3 minutes per sample.