Simultaneous Determination of (20 Amino Acids) Alanine, Arginine, Asparagine, Aspartic acid, Glutamic acid, Glutamine, Glycine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Ornithine, Phenylalanine, Proline, Serine, Threonine, Tryptophan, Tyrosine and Valine in K3EDTA Human Plasma by using LC-ESI-MS/MS.

Dr. Pritesh Contractor*, Dr Ajay Gupta, Ms Swati Guttikar, Mr. Ajit Amritkar, Mr Praveen sing Rao

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Introduction:

Amino acids are the building blocks of proteins and are additionally utilized as a source of energy. They are necessary for the synthesis of a wide variety of compounds, including neurotransmitters, haem and DNA. Humans need daily supplies of protein including adequate amounts of essential amino acids, which cannot be synthesized endogenously. Human body needs 20 different amino acids to grow and function properly. Though all 20 of these are important for your health, only nine amino acids are classified as essential. These are histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. There are nonessential amino acids that are classified as conditionally essential. Amino acid metabolomics in human serum has been studied on a larger scale due to its potential diagnostic value in patients with breast, lung, ovarian, head and neck, gastric, and pancreatic cancers, suggesting that the amino acid profiling in plasma or serum. Therefore, an accurate liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed and validated for the simultaneous determination of 20 amino acids in human plasma samples from healthy subjects. Multiple reactions monitoring mode was selected with electrospray ionization source operating in the positive ionization mode for data acquisition. Samples were prepared using specific precipitation technique followed by derivatization process using 50.0µL sample processing volume. Recovery, Matrix Factor, stability of analyte in blood and different stability studies has been performed which overall suffice to mimic the subject sample condition.

Sample Preparation:

Prior to analysis, blank samples, calibration curve standards and quality control samples are prepared. Blank samples and Calibration curve standards are prepared by using milli-q water as altered matrix. Quality control samples are prepared by using milli-q water and human plasma having anticoagulant as K3EDTA. QCs prepared by using human plasma are known as unaltered QCs and milli-q spiked QCs are known as altered QCs. After spiking unaltered QCs are diluted to five fold by using milli-q water.

Sample Preparation - Protocol

Aliquot 50.0µL sample (CC, QCs and subject samples), (plasma QCs & subject samples diluted x 5 fold → Add 50.0µL of Mix ISTD dilution (except BLK) followed by addition of 200.0µL of precipitation solution

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Vortex to mix and centrifuge the samples at 10000 rpm at 5±2°C for 10 minutes → Take 30.0µL of supernatant into pre-labeled tube and add 100.0µL of Buffer solution, pH 9.20 and vortex to mix

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Take 50.0µL of Derivatized samples and dilute with 1.00 ml water and vortex to mix ← Add 20.0µL of Derivatization Reagent, vortex to mix and Incubate the samples for 15 min at 60±5°C

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Transfer required volume of samples into auto-sampler vials and inject by using LC-FSI-MS/MS

Evaluation of Final QCs Concentration (for plasma QCs)

Nominal Concentration of QCs (HQC, MQC, LQC and LLOQ OCs) + Endogenous Content of Amino Acid, ZERO (e.c) (Mean of 3 Replicates) = Final Amended QCs Concentratrion for evaluation and acceptance of QCs

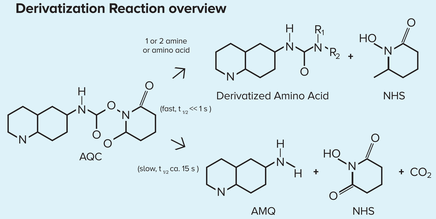
Calibration Curve Range:

Group	Amino Acid	ULOQ	LLOQ
Group 1	Alanine, Glutamic acid, Glutamine, Glycine, Histidine, Isoleucine, Leucine, Lysine, Ornithine, Phenylalanine, Proline, Threonine, Tryptophan, Tyrosine, Valine	20.000µg/mL	0.500µg/mL
Group 2	Asparagine, Methionine	4.000µg/mL	0.100µg/mL
Group 3	Arginine	80.000µg/mL	2.000µg/mL
Group 4	Aspartic acid	6.000µg/mL	0.150µg/mL
Group 5	Serine	40.000µg/mL	1.000µg/mL

Derivatization Overview:

AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) is an N-hydroxysuccinimide-activated heterocyclic carbamate, a class of amine-derivatizing compounds. Derivatization reagent converts both primary and secondary amino acids to stable derivatives, as illustrated in the figure below. The structure of the derivatizing group is the same for all amino acids. In a slower reaction, excess reagent hydrolyzes to produce 6-aminoquinoline (AMQ), N-hydroxysuccinimide (NHS) and carbon dioxide. The destruction of excess reagent is completed within 1 minute. NHS and carbon dioxide do not interfere with the analysis.

Derivatization Reaction overview



LC and Mass Parameters:

Column	Kinetex Polar C18 100 x 4.6mm,2.6µm
Mobile Phase	Pump A: Formic acid in water, 0.1%v/v Pump B: 0.1% Formic acid in Water: Acetonitrile (50:50v/v)
Initial Flow Rate	0.500 mL/min, B concentration: 4.00%,Gradient Mode
Column oven temperature	50 + 3°C
Auto sampler temperature	5 + 3°C
Volume of injection	20µL
Detector	Shimadzu SH 8040 Mass spectrometer
Run time	36.00 minutes

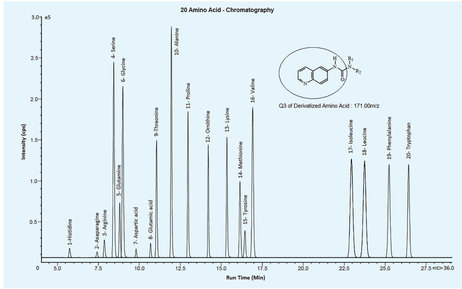
Mass Parameters:

Analyte Name	MRM Tansition	Q1 Pre Bias (V)	Collision Energy (CE)	Q3 Pre Bias (V)
20 Amino Acid	M.W. (derivatized) >171.00	-34 to -10	-29 to -12	-32 to -14

Isotope labelled Internal Standard (ISTD):

Alanine D4, Arginine D7, Asparagine D3, Aspartic Acid D3, Glutamic acid D5, Glutamine D5, Glycine D2, Histidine D3, Isoleucine D10, Leucine D3, Lysine D4, Methionine D3, Ornithine D7, Phenylalanine D7, Proline D3, Serine D3, Threonine D5, Tryptophan D3, Tyrosine D4 and Valine D8

Chromatographic Presentation:



Stability Assessment:

The matrix stability was evaluated by using freshly prepared duplicate calibration curve standard and two sets of freshly prepared batch qualifying QCs from Water & Unaltered matrix (at high and low level) along with six sets of stability samples in both Water and unaltered matrix (HQC and LQC levels).

Stability Condition	LQC (WaterSpiked)	HQC (Water Spiked)	LQC (Plasma Spiked)	HQC (Plasma Spiked)
	% Mean Bias			
Bench Top (BT) stability 07 Hours at Ambient Temperature	-0.04 to 11.72	-0.66 to -11.73	-13.00 to 2.29	-7.59 to 11.72
Freeze Thaw Stability 5 Cycles at freezing temperature of -20°C	-9.03 to 0.27	-7.41 to -1.69	-8.09 to 2.63	-9.11 to 0.19
Freeze Thaw Stability 5 Cycles at freezing temperature of -78°C	-2.72 to 3.20	-2.72 to 3.20	-5.12 to -0.53	-5.12 to -0.53
Stability of Extract (SE) 264 hours at 5±3°C in Water	-4.58 to -0.54	-4.58 to -0.54	-11.01 to -2.74	-11.01 to -2.74
Stability of Extract (SE) 03 hours at Ambient Temperature in Water	-8.62 to -0.49	-8.62 to -0.49	-11.06 to 1.85	-11.06 to 1.85
Long-Term Stability of Analyte in Matrix 76 Days at -20°C storage temperature	-7.30 to 3.90	-7.30 to 3.90	-14.51 to -4.81	-14.51 to -4.81
Long-Term Stability of Analyte in Matrix 76 Days at -78°C storage temperature	-4.77 to 8.33	-4.77 to 8.33	-12.81 to 2.07	-12.81 to 2.07

Validation Experiments:

Selectivity of Method in Presence of Concomitant Medication Drugs:

	% Mean Bias	
	CME HQC	CME LQC
Results Summary (In Presence of Acetaminophen, Caffeine, Cetirizine, Domperidone, Diclofenac, Ibuprofen, Nicotine and Ranitidine)	0.58 to 9.83	-11.12 to 0.32

Recovery - Analyte & Internal Standard

% Recovery = (Individual Extracted Peak Area / Overall Mean Unextracted Peak Area) × 100	92.00 to 111.00 % (Overall at HQC, MQC and LQC Level)

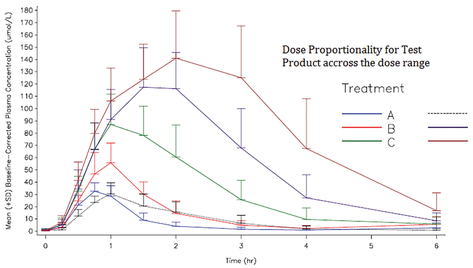
Matrix Effect by evaluating Matrix Factor	HQC	LQC
Matrix Factor for Analyte (Mean)	0.76 to 0.93	0.81 to 0.98
ISTD Normalized Matrix Factor (Mean)	0.92 to 1.03	0.96 to 1.40
% CV of ISTD Normalized Matrix Factor	1.84 to 7.40	4.93 to 12.23

Stability of Analyte in Blood	HQC	LQC
For 02 hrs. at wet ice bath (below10°C) and Ambient Temperature with respect to zero hour.	% Mean Stability within 85.00-115.00% and Precision within 15.0%	

Application to Study Sample Analysis and ISR:

This study was designed to evaluate safety/tolerability and pharmacokinetics of test product. The study provides key information on, dose proportionality of test product across the different dose range vs. the reference product (i.e. protein shake). The results of this study will support dose selection for future investigations.

Representative Pharmacokinetic Profiles



Incurred sample reanalysis (ISR) is commonly defined as the reanalysis of a subset of clinical study samples during sample analysis in order to demonstrate the perceived robustness of previously validated bioanalytical methods.

Total No. of Subject Sample analysed : 740 Samples
Sample Selected for ISR : 74
ISR Acceptance : 93.0% (average of all 20 Amino acids)

Conclusion:

The method was successfully validated and was selective, precise, accurate, robust including all required stability experiments. The procedures have been applied to clinical analysis of pharmacokinetic studies of constituent amino acids within test product. The acceptance of incurred sample reanalysis (i.e. more than 93.0%) indicates the assay is reproducible and robust.

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2. AccQ-Tag Ultra Derivatization kit care and use manual. t
3. US FDA - Bioanalytical Method Validation Guidance for Industry, May 2018

Block 6, Magnet Corporate Park 100 ft. Rhaltej - Hebatpur Road, Nr. Sola Bridge, Off. S.G. Highway, Thaltej, Ahemdabad - 380054

info@veedacr.com **www.veedacr.com**

***Corresponding Author**