Determination of the amino acids as biomarkers for metabolic disorders

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Introduction

The aim of the study was to develop and validate a method for the determination of the amino acids homocysteine, valine, methionine, allo-isoleucine, isoleucine, leucine, tyrosine and phenylalanine of the amino acids in human blood plasma in one run. Maple syrup urine disease (MSUD) is an autosomal recessive metabolic disorder affecting amino acids Valine, allo-Isoleucine, Isoleucine and Leucine. And increased level of homocysteine associated with homocystinuria. another metabolic disease. Phenylketonuria (PKU) is a rare inherited disease in which the body cannot metabolize the amino acid phenylalanine. Normally it will be metabolized and convert to the amino acid tyrosine. High levels of phenylalanine are harmful to the brain.

Sample Preparation and Analysis

500 µl blood plasma and 100 µl 4% dithiothreitol solution were mixed in a 1.5 mL tube and incubated by 40° for 30 min. Bound forms of homocysteine in the sample are reduced in form of free homocysteine by the use of 4% dithiothreitol solution. After the incubation 150 µL of precipitation solution were added and deposit in the refrigerator for 20 min for the protein precipitation. Then 500 µL of sample dilution buffer (including internal standard norleucin. 100 nmol/mL) were added and mixed. The supernatant was filtered with a membraSpin by centrifugation at 14000 rpm for five minutes. The particle free solution was used for the injection.

The samples were analyzed by the Amino Acid Analyzer ARACUS. manufactured and distributed by membraPure GmbH worldwide. ARACUS is using the classic routine analysis of amino acids by post-column derivatization with ninhydrin and the detection at 440 nm and 570 nm.

Figure 1: Amino Acid Analyzer ARACUS

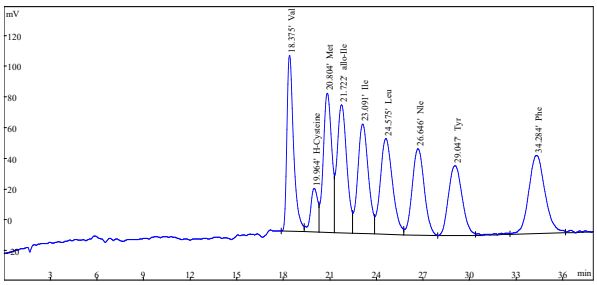

Figure 2: Chromatogram of Standard.

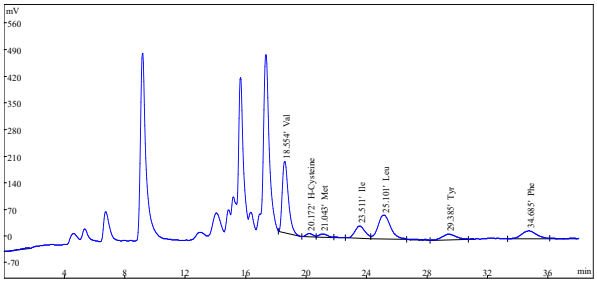

Figure 3: Chromatogram of blood plasma sample. used in this application for validation and presicion studies.

Results & Discussion

The Table 1shows the results from newborns without MSUD. The Table 2 shows the results from newborns with MSUD.

Table 1: Amino acid concentration of valine (Val). homocysteine (H-Cystein). methionine (Met). allo-isoleucine (allo-Ile). Isoleucine (Ile). Leucine (Leu). Tyrosine (Tyr). and Phenylalanine (Phe) in nmol/mL plasma of newborn without MSUD.

Amino Acid	$\bar{x}$	SD
Val	202.1	± 54.1
H-Cystein	4.4	± 1.5
Met	31.1	± 7.9
allo-Ile	< 2	-
Ile	58.9	± 16.9
Leu	141.9	± 40.5
Tyr	108.1	± 31.2
Phe	99.4	± 18.6

Table 2: Amino acid concentration of valine (Val). homocysteine (H-Cystein). methionine (Met). allo-isoleucine (allo-Ile). Isoleucine (Ile). Leucine (Leu). Tyrosine (Tyr). and Phenylalanine (Phe) in nmol/mL plasma of newborn with MSUD.

Amino Acid	Mean $\bar{x}$	SD
Val	467.9	± 116.9
H-Cysteine	6.9	± 2.8
Met	17.2	± 4.0
allo-Ile	66.2	± 27.4
Ile	213	± 48.6
Leu	433.6	± 108.1
Tyr	67.4	± 20.9
Phe	70.2	± 11.2

The results show the concentration levels of the amino acids valine, allo-isoleucine, isoleucine and leucine increases by newborn with MSUD. The normal reference range for Homocysteine is 4.2 – 12.8 nmol/ml Plasma. An increased homocysteine concentration associated with Homocystinuria.

A positive newborn screening test for PKU must be followed up by performing quantitative plasma amino acid analysis. measuring the concentration level of phenylalanine and tyrosine. A phenylalanine value of blood plasma of greater than 360 µM is consistent with diagnosis of PKU. PKU can cause abnormally low levels of tyrosine, a nonessential amino acid.

Reference values of phenylalanine:

- Premature: 90-213 nmoL/mL
- 0-31 days: 38-137 nmoL/mL
- 1-24 months: 31-75 nmoL/mL
- 2-18 years: 26-91 nmoL/mL

Reference values of tyrosine:

- Premature: 147-420 nmoL/mL
- 0-31 days: 55-147 nmoL/mL
- 1-24 months: 22-108 nmoL/mL
- 2-18 years: 24-115 nmoL/mL

For this study currently no blood plasma of a newborn with PKU was available but the application note is shown with the method it is possible to analysis all key components which are associated with MSUD and PKU in one single run.

Validation/ Precision

The precision of the method was determined by repeatability (intra-day) (Table 3) and intermediate precision (inter-day) (Table 4). The intra-day precision was calculated as the relative standard deviation (RSD) of results from ten runs of the sample. during the same day. The inter-day precision was studied by a daily sample preparation for ten times over a

period of two weeks. The standard deviation (SD) and RSD were calculated.

Table 3: Intra-day precision of amino acids in blood plasma. concentration in mg/g (injection n=10).

Amino Acid	Mean $\bar{x}$	SD	RSD / %
H-Cys	7.34	± 0.305	4.15
Val	164.4	± 0.855	0.52
Met	20.87	± 0.244	1.17
Ile	46.22	± 0.269	0.58
Leu	107.2	± 0.586	0.55
Nle	99.53	± 0.607	0.61
Tyr	67.8	± 0.380	0.56
Phe	69.1	± 0.511	0.73

Table 4: Inter-day precision of amino acids in blood plasma. concentration in mg/g (injection n=10).

Amino Acid	Mean $\bar{x}$	SD	RSD / %
H-Cys	7.56	± 0.436	5.77
Val	163.8	± 2.172	1.33
Met	21.24	± 0.592	2.76
Ile	44.67	± 0.908	2.03
Leu	105.3	± 1.721	1.63
Nle	100.37	± 1.141	1.13
Tyr	69.13	± 0.830	1.2
Phe	70.35	± 0.617	0.88

Accuracy (Recovery)

In this validation study, accuracy of the method has been investigated by calculating the recovery values (Table 6) obtained by analyzing the plasma sample spiked with homocysteine standard (40 nmol/mL). For the stock solution for the serial dilution preparation 2 mL homocysteine standard were added to 2 mL blood plasma. The scheme of the serial dilution samples corresponding to 100%, 80%, 60%, 40%, 20% and 10% is shown in Table 5.

Table 5: Scheme of the serial dilution samples

Plasma sample concentration/ %	Plasma sample / µL	Sample dilution buffer / µL
100	1000	-
80	800	200
60	600	400
40	400	600
20	200	800
10	100	900

Linearity

The linearity is important for the confirmation of the method's sensitivity for the analysis of the amino acid concentration within a defined range.
Linearity is the ability to obtain test results which are directly proportional to the concentration of the amino acid. Linearity was determined by five injections of six different concentrations 5, 10, 20, 30, 40 and 50 nmol/mL. The average peak areas were plotted against concentrations. Then linearity was evaluated using the calibration curve to calculate coefficient of correlation. slope and intercept (Table 7). In general a value of correlation coefficient (R^2)•>•0.990 is considered as the evidence of an acceptable fit for the data to the regression line.

Table 6: Recovery values for dilution series for each amino acid, which were analysed in nmol/mL blood plasma (injection n=10).

blood plasma						
Run	**100%**	**80%**	**60%**	**40%**	**20%**	**10%**
H-Cys						
mean $\bar{x}$	43.48	35.21	26.0	17.15	8.77	4.26
SD	± 0.308	± 0.267	± 0.175	± 0.494	± 0.0898	± 0.0975
RSD %	0.71	0.76	0.67	2.88	1.02	2.29
Recovery %	-	80.98	59.8	39.43	20.23	9.79
Val						
mean $\bar{x}$	166.8	132.8	103.7	67.43	32.39	16.72
SD	± 0.739	± 0.83	± 0.452	± 0.256	± 0.12	± 0.148
RSD %	0.44	0.63	0.44	0.38	0.37	0.89
Recovery %	-	79.62	62.16	40.43	20.62	10.02
Met						
mean $\bar{x}$	21.99	17.4	13.62	8.95	4.54	2.21
SD	± 0.154	± 0.201	± 0.149	± 0.0368	± 0.051	± 0.0272
RSD %	0.154	1.16	1.09	0.41	1.12	1.23
Recovery %	-	79.12	61.94	40.7	20.65	10.05
Ile						
mean $\bar{x}$	44.75	35.55	27.84	18.4	9.5	4.79
SD	± 0.213	± 0.228	± 0.276	± 0.088	± 0.085	± 0.084
RSD %	0.48	0.64	0.99	0.48	0.89	1.75
Recovery %	-	79.44	62.21	41.12	21.23	10.71
Leu						
mean $\bar{x}$	109.3	87.36	68.06	44.27	22.56	11.05
SD	± 0.591	± 0.459	± 0.642	± 0.196	± 0.199	± 0.109
RSD %	0.54	0.53	0.94	0.44	0.88	0.98
Recovery %	-	79.54	61.97	40.31	20.54	10.6
Tyr						
mean $\bar{x}$	69.89	54.71	43.44	28.87	14.81	7.17
SD	± 0.412	± 0.346	± 0.442	± 0.154	± 0.223	± 0.145
RSD %	0.59	0.63	1.02	0.53	1.51	2.02
Recovery %	-	78.28	62.15	41.31	21.19	10.26
Phe						
mean $\bar{x}$	69.12	55.12	43.09	28.79	14.79	7.76
SD	± 0.474	± 0.382	± 0.438	± 0.152	± 0.151	± 0.125
RSD %	0.69	0.69	1.02	0.53	1.02	1.61
Recovery %	-	79.74	62.34	41.65	21.4	11.23

Table 7: Resulted linearity of plotting average peak area against different concentrations of amino acids.

Amino Acid	R^2
H-Cys	0.9983
Val	0.9997
Met	0.9998
Ile	0.9999
Leu	0.9999
Nle	0.9998
Tyr	0.9999
Phe	0.9998

Table 8: Limit of detection and limit of qualification for each amino acid, analysed in blood plasma.

Amino Acid	LOD nmoL/mL	LOQ nmol/mL
H-Cys	0.82	2.46
Val	0.74	2.22
Met	0.84	2.52
Ile	0.83	2.49
Leu	0.76	2.28
Nle	0.8	2.4
Tyr	0.76	2.28
Phe	0.89	2.27

LOD & LOQ

The limit of detection (LOD) is the smallest amount or concentration of the amino acid in the sample that can be reliable distinguished from zero.
Determine the mean value and standard deviation (SD) of ten independent measurements of a blank sample or of a sample with very low concentrations of the measurand.
Limit of detection LOD = $\bar{x}$ + 3 SD.

The limit of qualification (LOQ) is the lowest concentration of the amino acid that can be quantitatively determined with accuracy and precision under the fixed acceptance criteria.
Limit of quantitation LOQ = 3 LOD.

References

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- Schadewaldt P. et al Significance of L-allo-isoleucine in plasma for diagnosis of maple syrup urine disease. Clin Chem. 1999 Oct; 45(10):1734-40.

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