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Phenylalanine, Tyrosine & Tryptophan HPLC Assay

Catalog Number: PNL31-H100
100 Tests
For Research Use Only. Not for use in diagnostic procedures.
v. 1.0

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1. Intended purpose

The Eagle Biosciences Phenylalanine, Tyrosine & Tryptophan HPLC Assay kit is intended for the quantitative determination of phenylalanine, tyrosine and tryptophan in whole blood, plasma and filter spots. This Phenylalanine, Tyrosine & Tryptophan HPLC Assay Kit is for research use only and is not for use in diagnostic procedures.

2. Introduction

Phenylketonuria is the most abundant genetic disorder of the amino acid metabolism. It is characterized by a lack of phenylalanine hydroxylase which causes an increase of phenylalanine in cells and body fluids. Due to the lack of enzyme activity, phenylalanine, normally metabolised to tyrosine, is converted to phenylpyruvic acid. Left untreated, this can cause brain damage and progressive mental retardation. Additionally high concentrations of phenylalanine inhibit various enzymes which are involved in the synthesis of neurotransmitters and melanin. The incidence of occurrence is about 1 in 15000, varying widely in different populations.

Untreated children with phenylketonuria are normal at birth, but rather early they don´t attain early developmental milestones, show mental retardation, hyperactivity, convulsions, low pigmentation of the hair and alterations of the skin. An early diagnosis within the first days after birth is very important. Nevertheless the blood level of phenylalanine has to be controlled regularly because reduced levels can lead to proteolysis with disturbance of growth.

The Eagle Biosciences Phenylalanine, Tyrosine & Tryptophan HPLC Assay kit makes it possible to determine phenylalanine, tyrosine and tryptophan in an easy, fast and precise method. The Phenylalanine, Tyrosine & Tryptophan HPLC Assay kit includes all reagents for preparation and separation of the samples with exception of the columns (IC4000rp) and the controls (IC4000ko). Both can be supplied by Eagle Biosciences. Besides the complete test kits it is possible to order all components separately. Please request our single component price list.

3. Warnings and precautions

- All reagents of this Phenylalanine, Tyrosine & Tryptophan HPLC Assay kit are strictly intended for research use only.
- Phenylalanine, Tyrosine & Tryptophan HPLC Assay kit and column are concerted. Using alternative columns might cause in insufficient separation, resulting in false high results. The given test characteristics might not be fulfilled.
- Do not interchange the Eagle Biosciences Phenylalanine, Tyrosine & Tryptophan HPLC Assay kit components from different lots.
- Calibrator and controls contain human serum. It was tested and found negative for HBsAg, anti-HIV-1/2, and anti-HCV. No test can guarantee the absence of

HBsAg or HIV, and so all human serum based reagents in this kit must be handled as though capable of transmitting infection.

- The mobile phase contains acetonitrile and must be handled carefully. Acetonitrile is highly flammable and toxic if inhaled or contact the skin. It should be handled with gloves, eye protection, and appropriate protective clothing in a hood. Any spill should be wiped out immediately with copious quantities of water. Do not breathe vapor and avoid inhalation. In case of an accident or indisposition contact immediately a physician.
- Wear disposable gloves while handling specimens or kit reagents and wash hands thoroughly afterwards.
- Do not pipette by mouth.
- Do not eat, drink, smoke or apply makeup in areas where specimens or kit reagents are handled.
- Phenylalanine, Tyrosine & Tryptophan HPLC Assay kit reagents should not be used beyond the expiration date shown on kit label.
- Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera. During handling of all Phenylalanine, Tyrosine & Tryptophan HPLC Assay kit reagents, controls and serum samples observe the existing legal regulations.

4. Material Provided

Article no.	Component	Designation	Amount
IC4000lm	ELU	Mobile Phase (contains acetonitrile)	1 x 1000 ml
IC4000ka	CAL	Calibrator (lyoph.; Concentration is given on the data sheet)	1 x 3 ml
IC4000is	IS	Internal standard vials	100 pcs.

5. Additional special equipment

- 1.5 ml reaction tubes (Eppendorf)
- Centrifuge
- Various pipettes
- Isocratic HPLC system with UV-detector
- HPLC column Phenylalanine (IC4000rp)
- Vortex mixer

6. Reagent preparation

- Reconstitute the **calibrator (CAL)** in **3 ml** deionized water and mix for 15 min, divide the calibrator in several portions and store them at -20 °C. Avoid repeated freeze-thaw circles. The concentrations are given on the product specification and might have minor changes from lot to lot.
- All other Phenylalanine, Tyrosine & Tryptophan HPLC Assay kit reagents are stable at 2-8 °C, up to the date of expiry stated on the label.

7. Specimen

For blood withdrawal, collecting tubes with an appropriate anticoagulant (e.g. EDTA) should be used.

- **Whole Blood**
  The samples are stable for 48 hours at 2-8 °C. At -20 °C, the samples are stable for at least 30 days. For preparation, the samples should be at room temperature. They have to be mixed thoroughly (avoid repeated thawing and freezing).

- **Plasma**
  Centrifuge the whole blood samples just after collection. The plasma can be stored up to 3 days at 2-8 °C. At - 20 °C the plasma samples are stable for 30 days (avoid repeated thawing and freezing).

- **Filter Spots**
  Pipette 50 µl of blood on the filter spot. The surface has to be completely moistened with the sample. The filter spots should be completely dried at 20-25°C (needs 30-60 min.). The filter spots are now stable for 5 days at 20-25 °C.

8. Procedure

Principle of the Method

For the determination of Phenylalanine, Tyrosine & Tryptophan a sample preparation has to be performed. Therefore a precipitation solution, containing an internal standard, is added. After centrifugation the supernatant is injected into the HPLC system. The isocratic separation via HPLC at 30°C lasts 10 minutes. The chromatograms are recorded by a UV-detector. The quantification is performed with the delivered plasma calibrator; the concentration is calculated via the "internal standard method" by integration of the peak heights respectively areas.

Sample preparation

Whole blood and plasma

1. Pipette **50 µl** sample, CAL or CTRL into the sample vials. The sample vials contain the internal standard dissolved in precipitation solution.
2. Mix briefly (5 sec.) on a vortex mixer.
3. Leave the tubes for **15 minutes at 2-8°C** and centrifuge afterwards at 10.000g for 5 minutes.
4. Inject **20 µl** of the supernatant for chromatography into the HPLC-system.

Filter spots

1. Put the filter spot (with dried patient blood) in a sample vial. The sample vials contain the internal standard dissolved in precipitation solution.
2. Mix briefly (5 sec.) on a vortex mixer.
3. Incubate the tubes for **30 minutes at 37°C,** leave them for 10 min in an ultrasonic bath and centrifuge afterwards at 10.000g for 5 minutes.
4. Inject **20 µl** of the supernatant for chromatography into the HPLC-system

Stability of the prepared sample:
The supernatant is stable for at least 5 hours at 20-25 °C, 24 hours at 2-8 °C and 10 days at -20 °C.

Chromatographic settings

Column material:	Eagle column (IC4000rp)
Column dimension:	125 mm x 4 mm
Flow rate:	1.0 ml/min
UV-detection:	210 nm
Injection volume:	20 µl
Running time:	10 min
Temperature:	30 °C

Treatment of the HPLC-column

After the analysis the column should be flushed with 20 ml 85% acetonitrile in deionized water (flow 1.0 ml/min). Before use, the system should be equilibrated with ca. 30 ml ELU.

9. Calculation of analytical results

Calculation

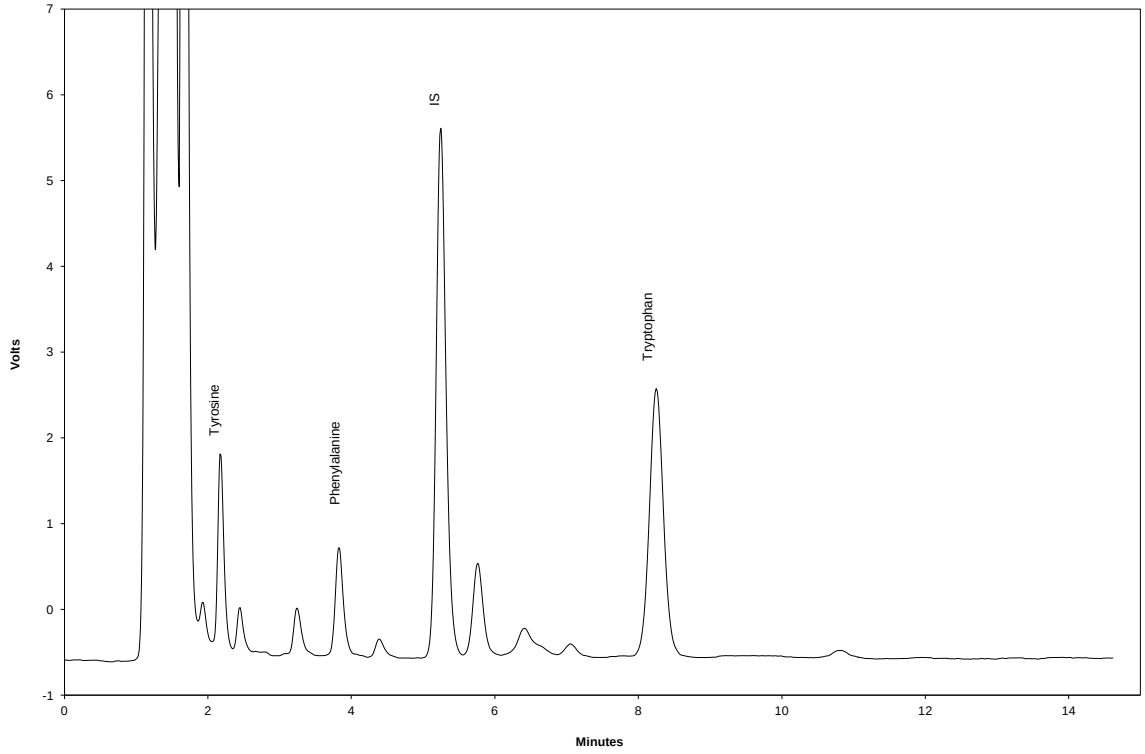
$$\text{Conc. sample}\,(mg/\text{dl}) = \frac{\text{peak area patient} * \text{conc. calibrator}\,(mg/\text{dl})}{\text{peak area IS patient}} * F$$

$$F = \frac{\text{Peak area IS of the calibrator}}{\text{Peak area analyte of the calibrator}}$$

Conversion to molarity:

Analyte	Molecular weight	Conversion
Phenylalanine	165.2	mg/dl x 60.5 = µmol/l
Tyrosine	181.2	mg/dl x 55.2 = µmol/l
Tryptophan	204.2	mg/dl x 49.0 = µmol/l

Typical chromatogram



Tyrosine	2.1 min
Phenylalanine	3.8 min
Internal standard	5.2 min
Tryptophan	8.2 min

10. Internal Quality Control

Reference intervals

Analyte	Matrix		Range [mg/dl]	Range [µmol/l]
Phenylalanine [4]:	Plasma:	Prematures:	2.0 – 7.5	121 – 454
		Neonates:	1.2 – 3.4	73 – 206
		Adults:	0.8 – 1.8	48 – 109
Tyrosine [4]:	Plasma:	Prematures:	7.0 – 24.0	386 – 1325
		Neonates:	1.6 – 3.7	88 – 204
		Adults:	0.8 – 1.3	44 – 72
Tryptophan [5]:	Plasma:	Neonates:	0.9 – 1.7	44 – 83
		Adults:	1.1 – 2.1	54 – 103

We recommend that each laboratory develop their own normal range. The values mentioned above are only for orientation and can deviate from other published data.

11. Validation data

Precision and reproducibility

Intra-Assay CV:

Tyrosine	1.3 % (51.5 µmol/l)	0.9 % (236.7 µmol/l)	[n = 6]
Phenylalanine	1.0 % (51.9 µmol/l)	0.5 % (270.1 µmol/l)	[n = 6]
Tryptophan	0.8 % (43.9 µmol/l)	0.6 % (217.4 µmol/l)	[n = 6]

Inter-Assay CV:

Tyrosine	3.0 % (52.0 µmol/l)	3.0 % (238.5 µmol/l)	[n = 6]
Phenylalanine	3.4 % (51.9 µmol/l)	3.3 % (271.3 µmol/l)	[n = 6]
Tryptophan	3.2 % (44.4 µmol/l)	3.7 % (218.9 µmol/l)	[n = 6]

Linearity

up to 2450 µmol/l (Tryptophan)

Detection limit

Tyrosine	0.5 µmol/l
Phenylalanine	0.5 µmol/l
Tryptophan	0.25 µmol/l

Recovery

Tyrosine	99.3 %
Phenylalanine	98.3 %
Tryptophan	96.9 %

12. Limitations of the method

Whole blood has a slightly higher variation.

13. Disposal

The mobile phase (ELU), internal standard and precipitation reagent must be disposed as non-halogenated solvent. Please refer to the appropriate national guidelines.

14. Troubleshooting

Problem	Possible reason	Solution
No signal	No or defect connection to evaluation system	Check signal cord and connection
	Detector lamp is altered	Change lamp
No peaks	Injector is congested	Check Injector
Double peaks	Dead volume in fittings and / or column	Renew fittings and / or column
Contaminating peaks	Injector dirty	Clean injector
	Contamination at the head of the column	Change direction of the column and rinse for 30 min at low flow rate (0.2 ml/min) with mobile phase
	Air in the system	Degas pump
	Autosampler vials contaminated	Use new vials or clean them with methanol
Broad peaks, tailing	Precolumn / column exhausted	Use new precolumn / column
Variable retention times	Drift in temperature	Use a column oven
	Pump delivers imprecise	Check pump, degas the system
	System is not in steady	Rinse system mobile phase

	state yet	for 15 min
Baseline is drifting	Detector lamp did not reach working temperature yet	Wait
	Detector lamp is too old	Renew lamp
Continue baseline is drifting	System is not in steady state yet	Rinse system mobile phase for 15 min
	Pump delivers imprecise	Check pump, degas the system
Baseline is not smooth	Pump delivers imprecise	Check pump, degas the system
	Detector flowcell is dirty	Clean flow cell

15. Literature references

1. F. Güttler, H. Lou in: C. J. Fernandez, J. M. Saudubray, G. van den Berghe (Hrsg.), Inborn Metabolic Diseases, 3rd Edition, , Springer Verlag, Berlin/Heidelberg/New York, 2000, p. 161-174.
2. S. Halvorsen in: C. J. Fernandez, J. M. Saudubray, G. van den Berghe (Hrsg.), Inborn Metabolic Diseases, 3rd Edition, , Springer Verlag, Berlin/Heidelberg/New York, 2000, p. 199-209.
3. D. Matthias, M. Böttcher, J. Gebhard, Quality Control in Neonatal Screening for Inborn Errors of Metabolism, Clin. Lab. 44 (1998), 51-60.
4. N. W. Tietz (Hrsg): Fundamentals of Clinical Chemistry, 4th Edition, Saunders 1996.
5. Leistungsverzeichnis der Laborarztpraxis Dr. Froreich u. Kollegen, Hamburg , 5. Aufl., 1996.

For further information about this kit, its application or the procedures in this insert, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.