Quantitative amino acids analysis for the diagnosis and follow up of inborn errors of metabolism

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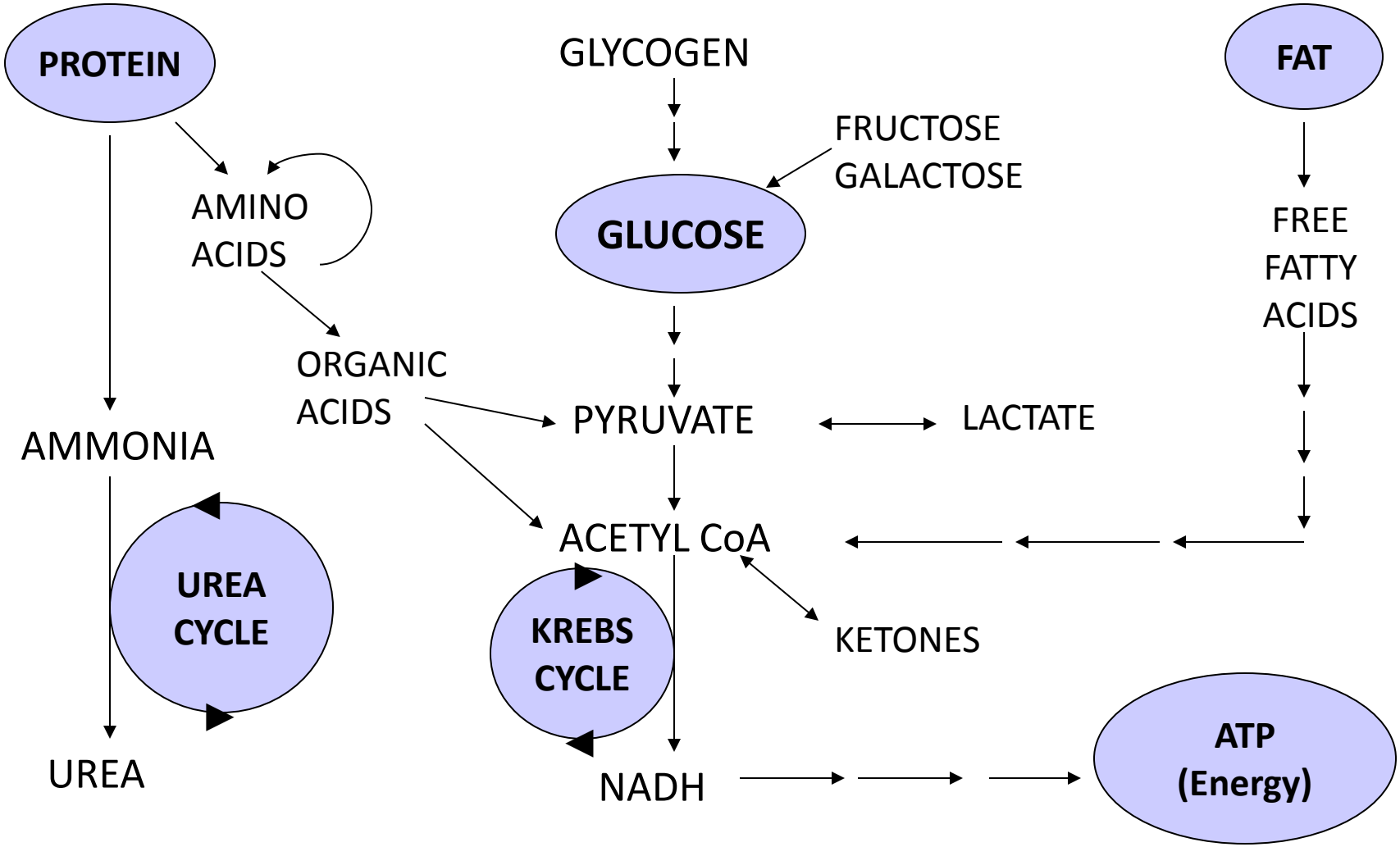
Conflict of Interest

- None to declare

Learning objectives

- Define Inborn Errors of Metabolism (IEM)
  - Emphasis on disorders of amino acid metabolism and transport, and urea cycle disorders
- Compare strengths and weaknesses among methods used to quantify physiological amino acids in body fluids
- Evaluate the use of quantitative amino acid analysis for IEM diagnosis and follow-up

Metabolism is sum of all chemical reactions that occur within an organism

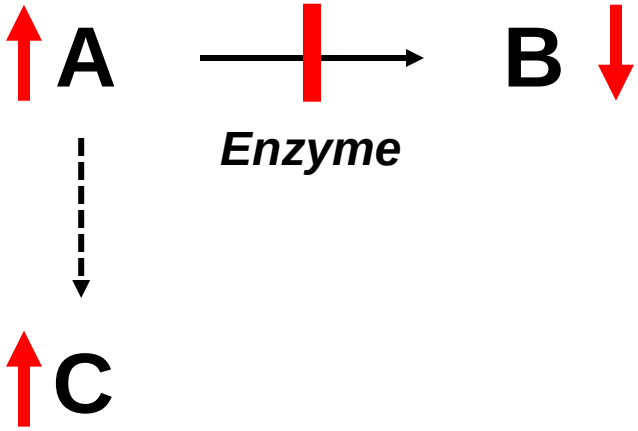


Pediatr Rev (1995) 16(10):390-5

Inborn errors of metabolism (IEM)

Genetic disorders affecting metabolic pathways

- Clinical signs and symptoms are caused by substrate accumulation, product deficiency, and/or alternative toxic byproducts



Several mechanisms can contribute to the metabolic block in IEM

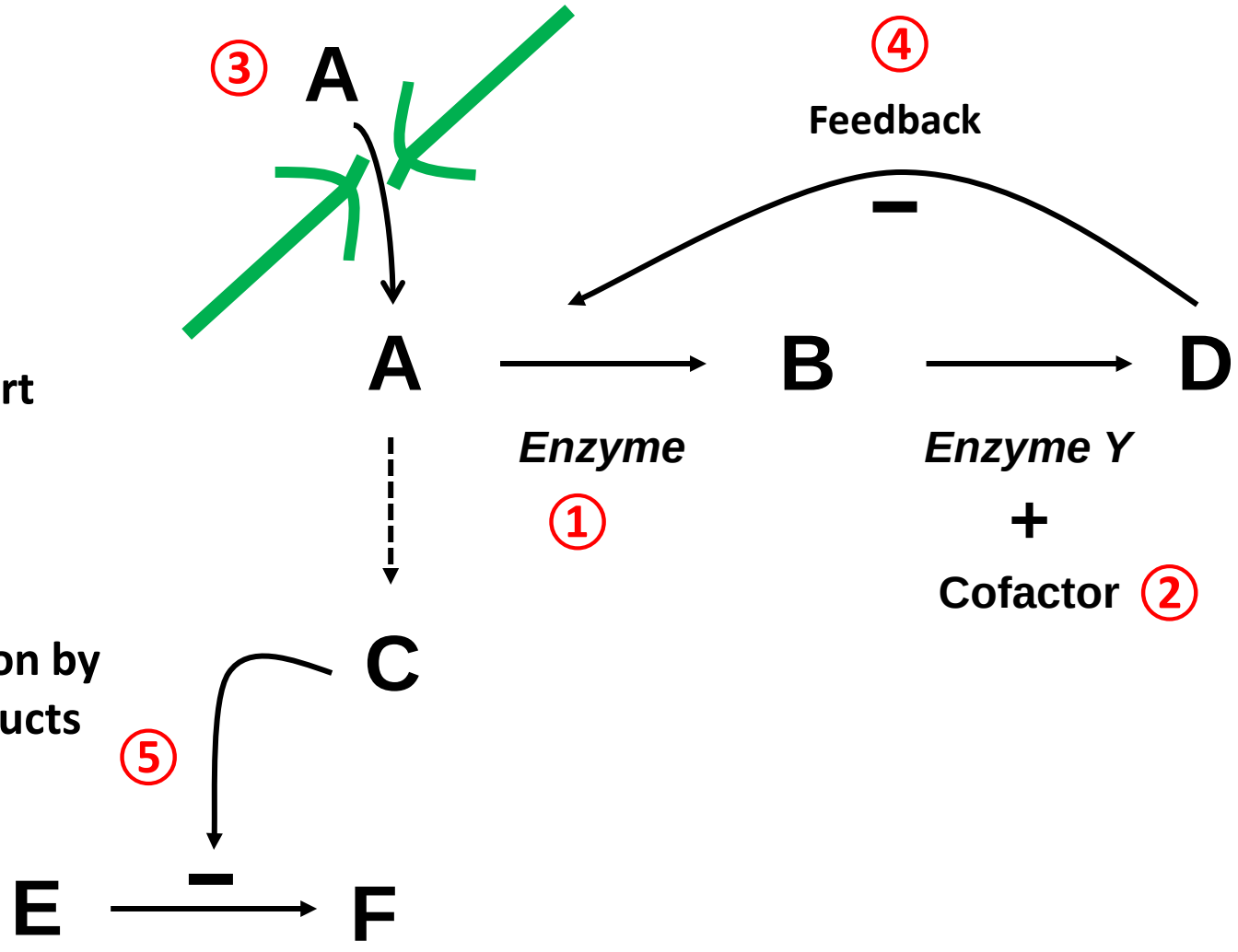
① Enzyme defect

② Cofactor defects

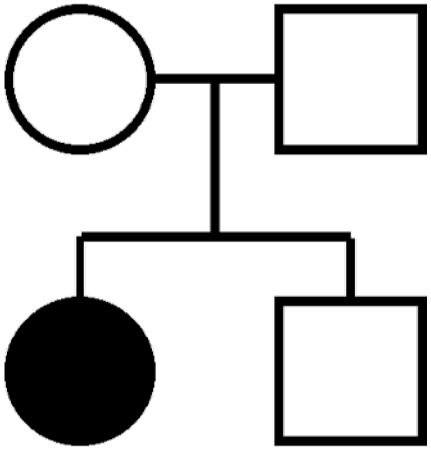
③ Decreased transport across membranes

④ Lack of feedback

⑤ Secondary inhibition by alternative byproducts



Most IEM are inherited as autosomal recessive disorders



- Heterozygotes do not show any clinical manifestations
  - Mating between two heterozygotes has a 25% chance to produce an affect child

IEM cumulative frequency is high approximately 1:2,000

- Individually, IEM are rare
  - PKU (phenylketonuria) 1:12,000
  - Tyrosinemia Type I 1:100,000
  - Homocystinuria 1:120,000
  - Citrullinemia Type I 1:150,000
  - Maple Syrup Urine Disease 1:180,000
  - Argininosuccinic aciduria 1:300,000

Clinical features suggestive of IEM may present at any age

- Acute, life-threatening illness
  - Poor feeding, vomiting, lethargy, progressing to seizures and coma
- Static or progressive disease
  - Hypo/hypertonia, seizures, developmental delay, movement abnormalities
  - Cardiomyopathy
  - Hepatocellular dysfunction
- Chronic, non-specific symptoms
  - Failure to thrive
  - Unusual odor

Most IEMs are treatable disorders

Treatment options

- Substrate restriction and/or product supplementation
- Enzyme's cofactors
- Stimulation of alternate pathways
- Enzyme replacement therapy
- Organ transplantation

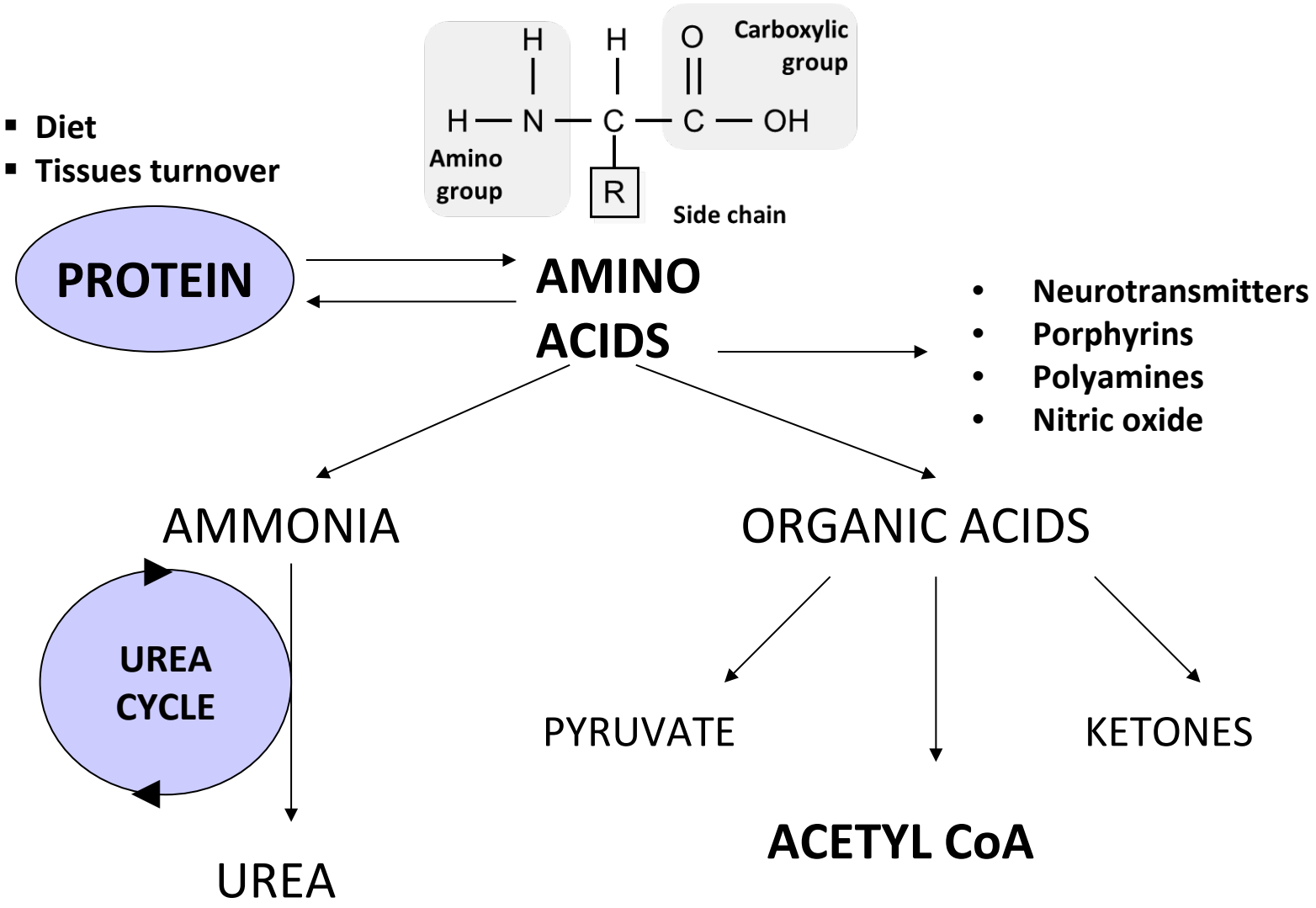
The importance of being tested

- Routine Laboratory Studies
  - Blood pH
  - Blood lactate and pyruvate
  - Plasma electrolytes
  - Plasma ammonia
  - Plasma glucose
  - Urine ketones
  - Liver function studies
  - Serum creatine kinase
- Biochemical Genetics Studies
  - **Amino acids (plasma, urine, CSF)**
  - Urine organic acids
  - Plasma carnitine & acylcarnitines
  - Urine acylglycines
  - Urinary oligosaccharides and glycosaminoglycans

Clinical indications for amino acids analysis

- Diagnosis of inborn errors of amino acid metabolism and transport
- Diagnosis of inborn errors of the urea cycle
- Diet monitoring in patients with known IEM
- Nutritional assessment of patients with non-metabolic conditions [e.g. short bowel syndrome]

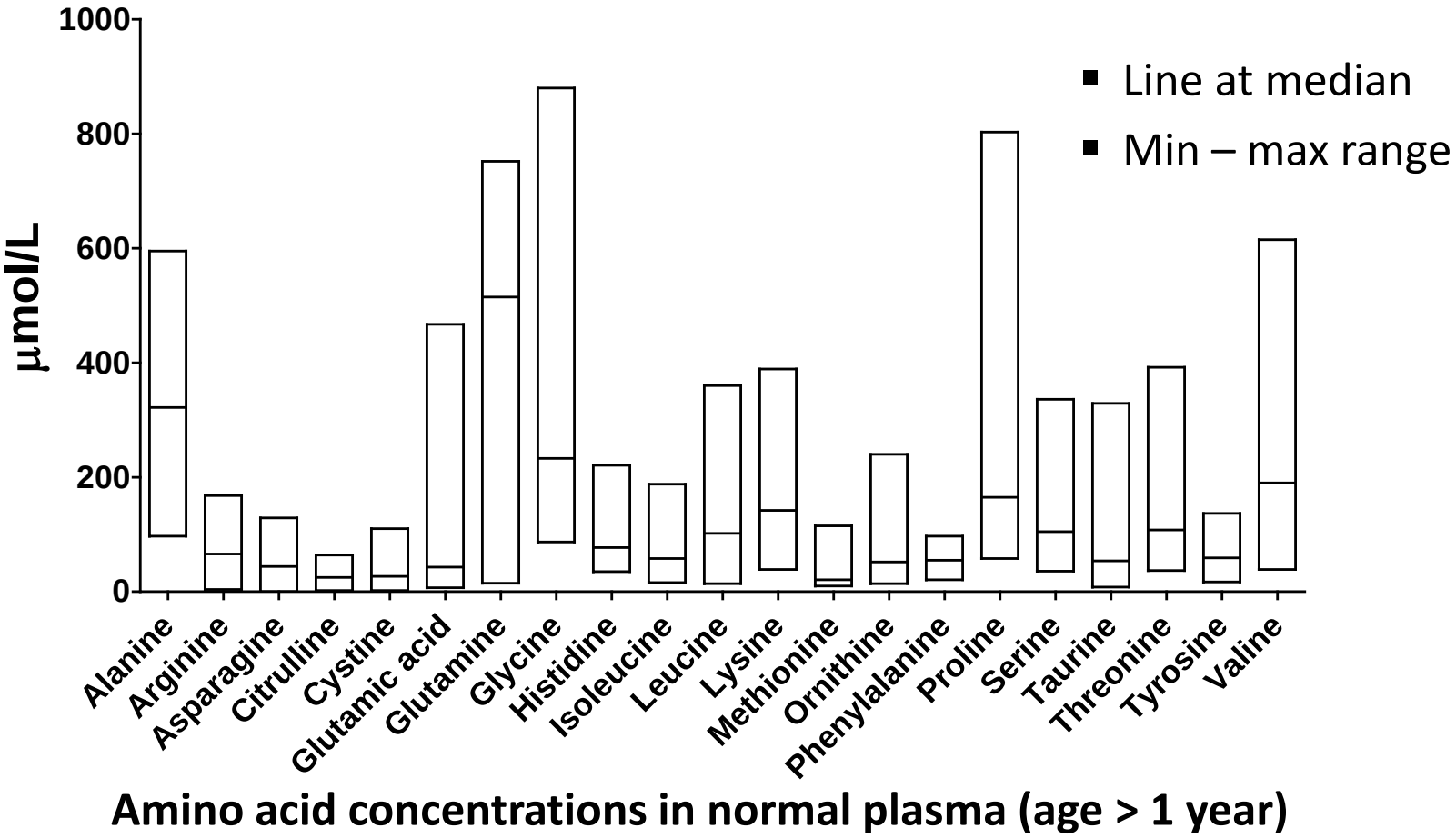
Amino acids function as structural units of proteins, source of energy and precursors



Modified *Pediatr Rev* (1995) 16(10):390-5

Some amino acids are physiologically more abundant

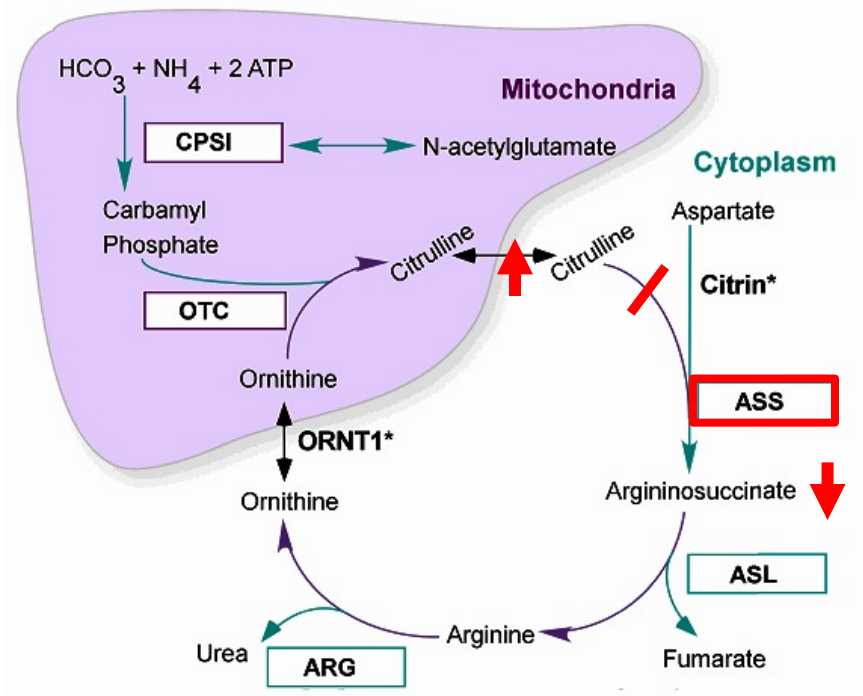
- Broad range of amino acids concentrations



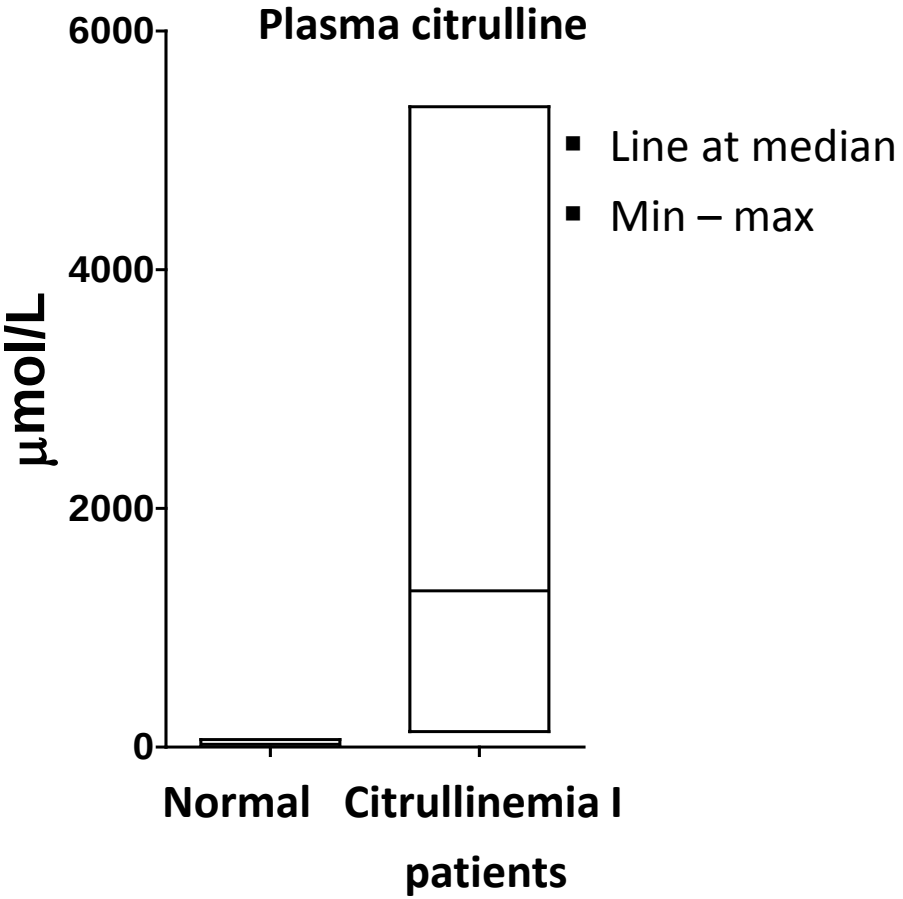
Amino acid concentrations in normal plasma (age > 1 year)

Amino acid abnormalities can indicate an inborn error of metabolism

- Disorders of amino acid metabolism and transport
- Urea cycle disorders

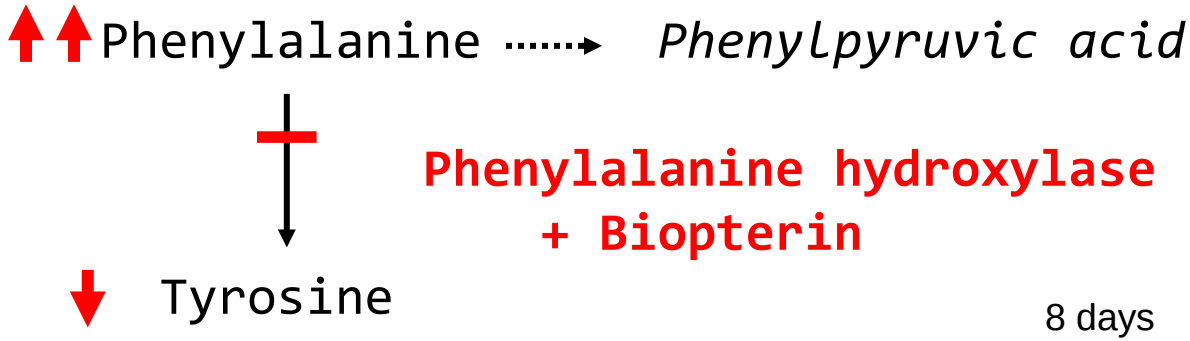


Urea Cycle Disorders Overview, GeneReviews



Amino acids patterns are characteristic of specific disorders

- Example phenylketonuria versus liver dysfunction

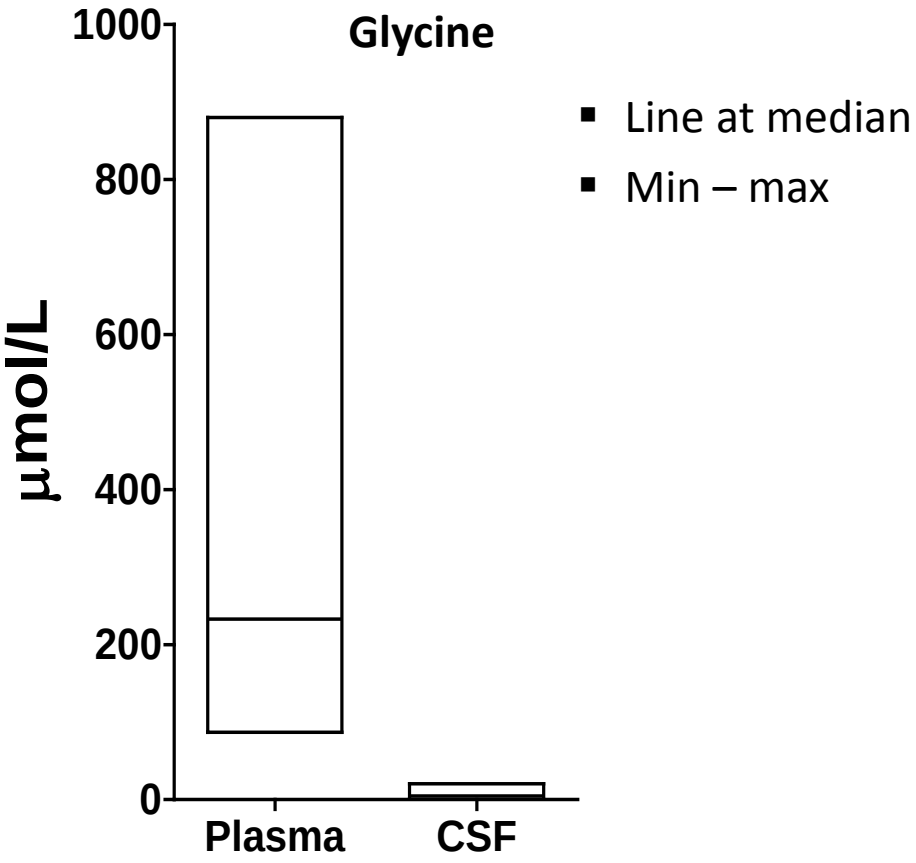


	8 days old girl		7 days old boy	
Citrulline	11	N	96	H
Lysine	174	N	2166	H
Methionine	29	N	796	H
Phenylalanine	1567	H	1139	H
Threonine	163	N	991	H
Tyrosine	28	L	517	H
Phe/Tyr ratio	56	H	2.2	N

Sample types used for clinical testing

- Amino acids abundance differ among sample types

  - **Plasma**
  - Cerebrospinal fluid (CSF)
  - Urine



Factors affecting amino acids analysis

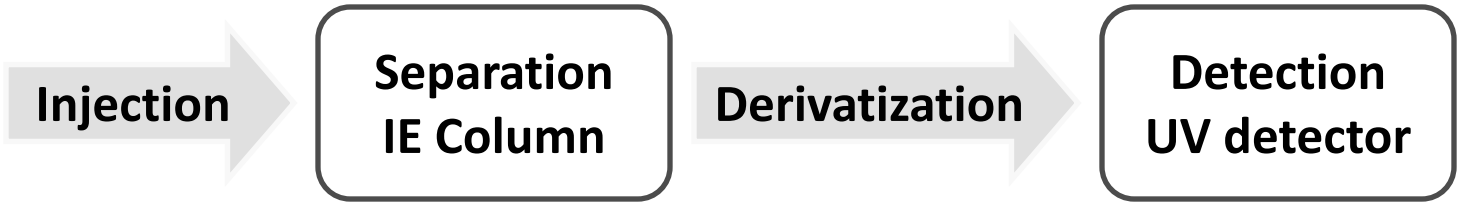
- Age, diet, medications
- Medications and other contaminants
  - Bacterial contamination (urine) increases alanine, glycine, proline and decreases in aromatic amino acids
  - Blood in CSF increases amino acids not specifically
- Storage temperature and time
  - Loss of cystine and homocystine (binding to plasma protein)
  - Loss of glutamine with increase in glutamic acid
- Hemolysis
  - Increase in glutamate, aspartate, taurine (high intracellular levels)
  - Increase in ornithine and decrease in arginine (release of the enzyme arginase from red cells)

Quantitative amino acids analysis

- Stanford Moore (left) and William Stein (right) about 1965 in front of the original amino acid analyzer
  - Courtesy of the Rockefeller Archive Center
- Ion-exchange chromatography with post-column ninhydrin detection

***Protein Science* (1993) 2:1188-91**

Ion Exchange Chromatography is still the gold standard for amino acids analysis



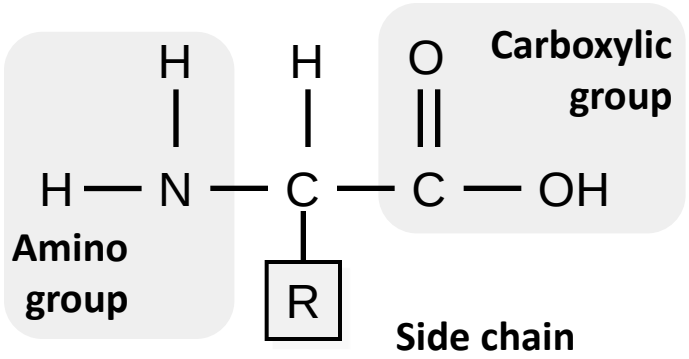
- Samples are de-proteinized with sulfosalicylic acid prior to injection
- Utilizes a lithium-based cation-exchange column
- Eluting amino acids undergo post column reaction with ninhydrin and subsequent optical detection

Biochrom 30 Analyser
Biochrom, UK

Amino acid concentrations are proportional to ninhydrin/eluate color

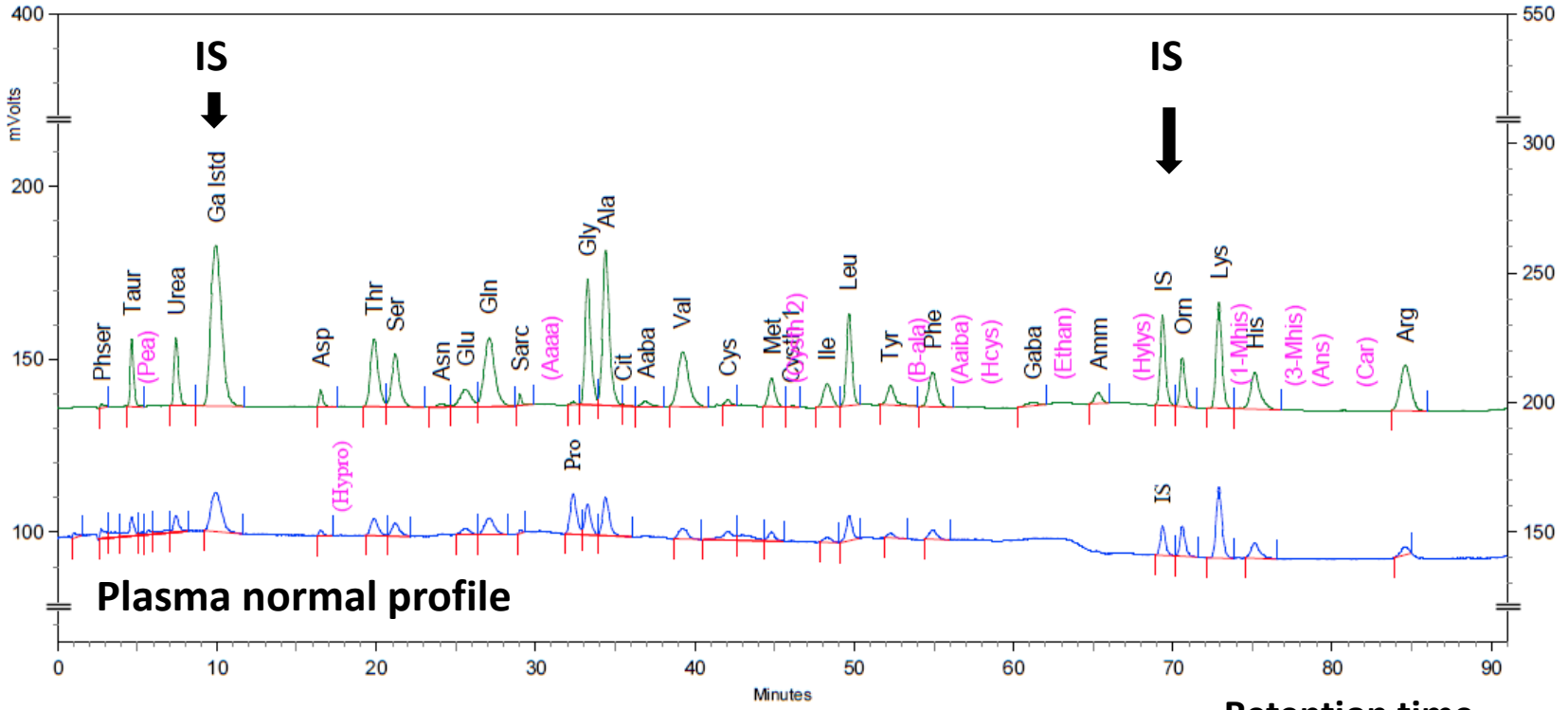
- Optical detection in the visible spectrum
  - amino acids: 570nm; imino acids 440 nm

Amino acids chromatogram is used for the determination of amino acid composition

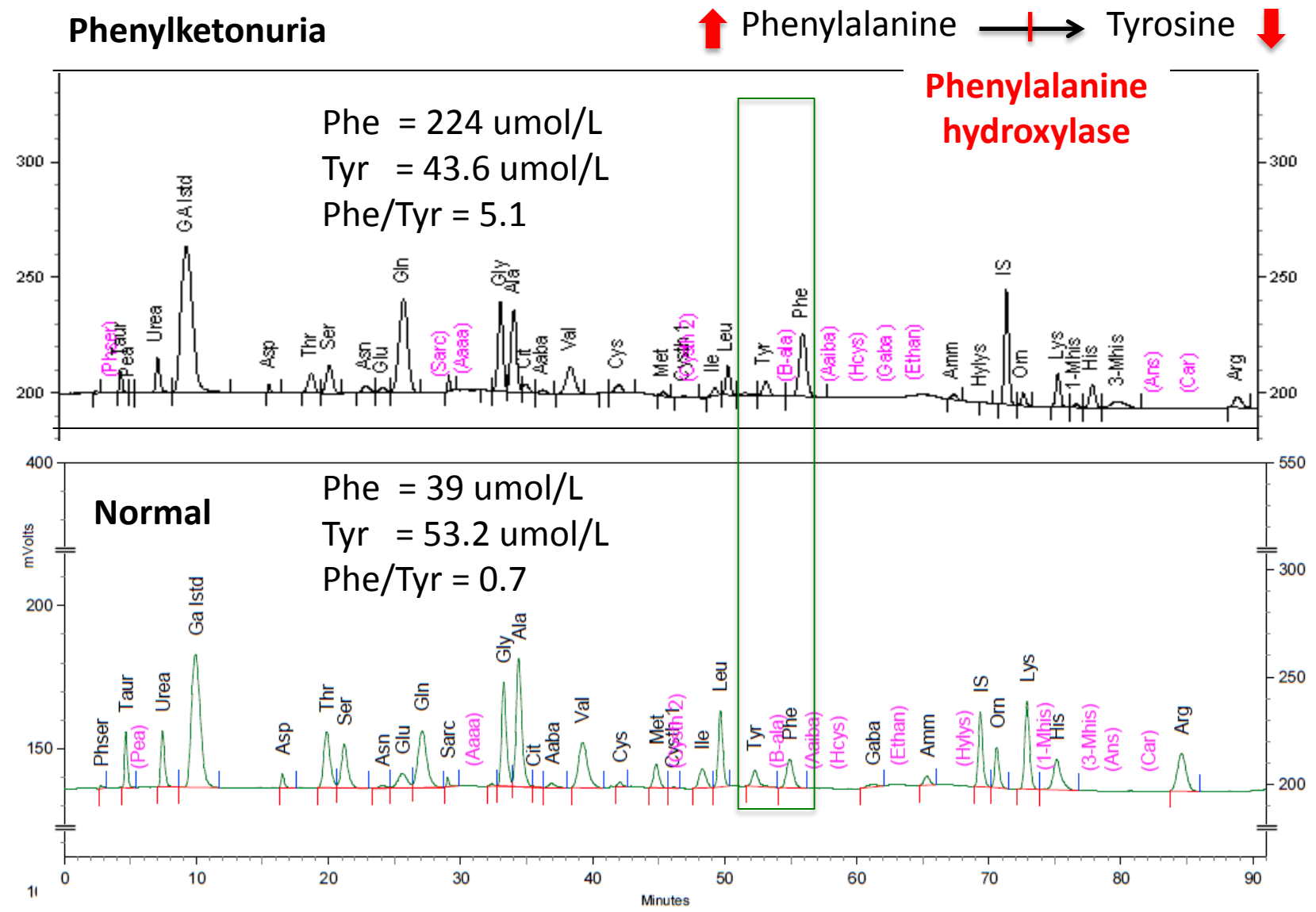


Factors influencing separation

- Side chain charge at neutral pH
- Temperature
- Ionic strength of the buffers



IEC can be successfully used for the diagnosis and follow-up of IEM

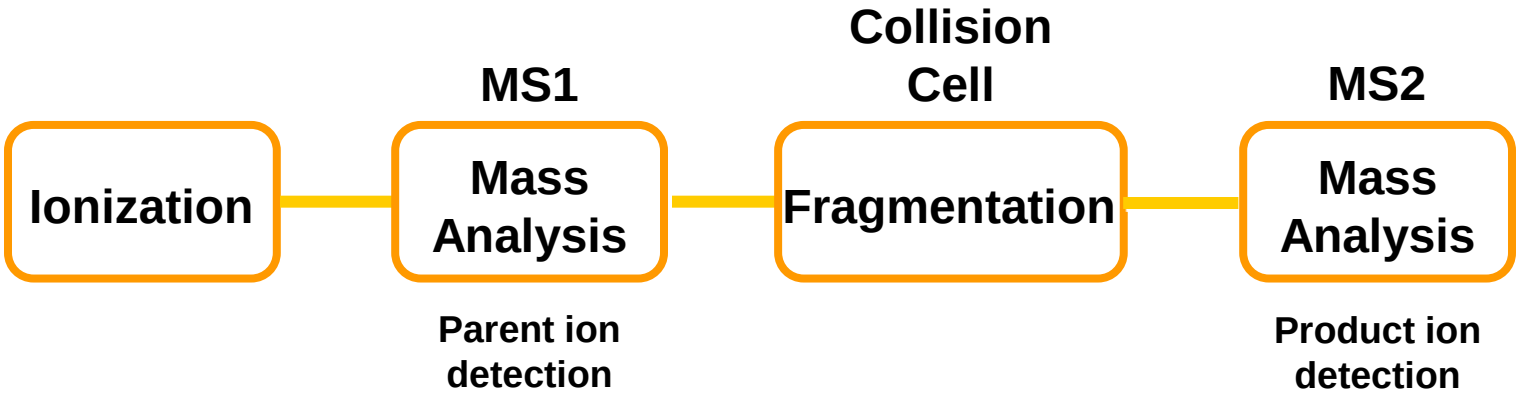


Disadvantages of Ion Exchange Chromatography

- Long run time (90 – 150 minutes)
- Lack of analyte specificity (identification by retention time)
- Co-eluting substances cannot be separated and distinguished

Alternative methods using mass spectrometry increase specificity

- Mass Spectrometry measures the ratio of the mass (m) of a chemical to its charge (z)
- Tandem Mass Spectrometry (MS/MS) combines two mass spectrometers



Mass spectrometry is routinely used for quantitative amino acids analysis

- MS/MS is used for the “expanded” newborn screening
  - High throughput
  - Short run time
- Combined with gas chromatography (GC-MS) or liquid chromatography (LC-MS/MS) is used for amino acids profiling in IEM
  - Isomers/isobars can be resolved

GS-MS and LC-MS/MS for quantitative amino acids analysis

- *Piraud et al. 2005 Rapid Commun. Mass Spectrom.*
  - **ion-paring liquid chromatography & MS/MS**
  - **derivatized amino acids (tridecafluoroheptanoic acid)**
- *Kaspar et al. 2008 Journal of Chromatography B*
  - **gas chromatography–mass spectrometry (GC–MS)**
  - **derivatized amino acids (propylchloroformate)**
- *Waterval et al. 2009, Clin Chim Acta*
  - **ultra-performance liquid chromatography (UPLC) & MS/MS**
  - **underivatized amino acids**
- *Kaspar et al. 2009 Journal of Chromatography B*
  - **ion-paring liquid chromatography & MS/MS**
  - **derivatized amino acids (TRAQ® reagents)**

The good, the bad and the ugly

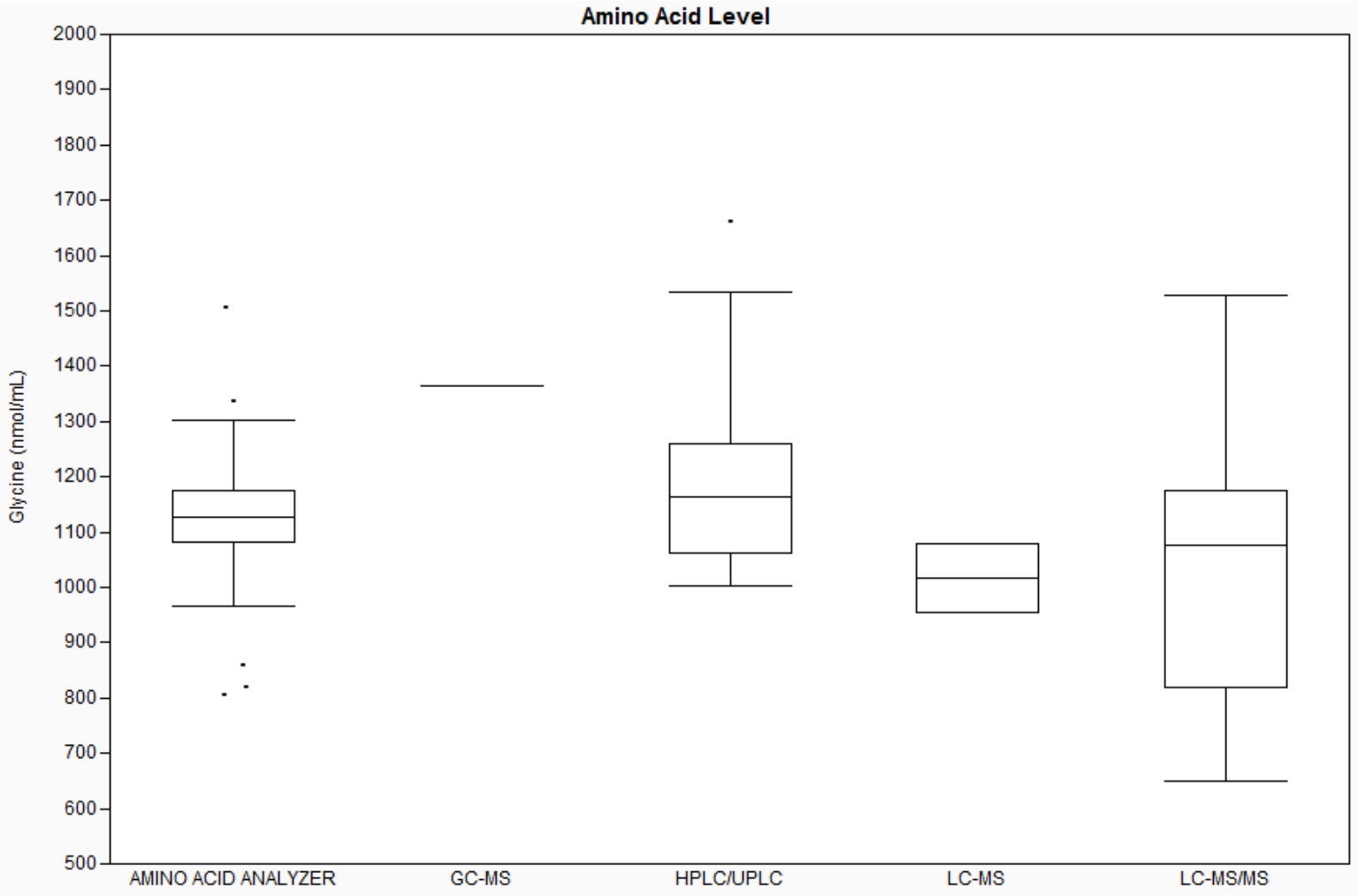
MS/MS	• Isomers/isobars cannot be resolved
GC-MS	• Not suitable for thermolabile amino acids derivatives
UPLC	• Poor quantification of phosphorylated amino acids • Ion suppression
IP-LC-MS/MS	• Labeled internal standards not available for all amino acids • Contamination with IP reagents
IP-LC-MS/MS using TRAQ reagents	• Poor recovery of sulfur containing amino acids (improved with new reagents)

Anal Bioanal Chem (2009) 393:445–452

Availability of internal standards

- Internal standards correct for matrix effects
  - Isotopically labeled version of the analyte
    - Not all standards commercially available
  - Tag-labeled version of the analyte
    - TRAQ™ reagents tag the amino groups of amino acids and amino acid-related compounds

Reproducibility among the different methodologies: cap survey



Amino acid proficiency testing, College of American Pathologists survey

Quantitative amino acids analysis
Analytical challenges

- Large number of analytes analyzed in a single run
  - > 40 physiological amino acids and related compounds
- Broad linear dynamic range
- High analyte sensitivity
- Analyte specificity
  - Separation of isobars/isomers

Comparison with Ion Exchange Chromatography

	Compound	R^2	Slope
1	α-Aminobutyrate	*	*
2	β-Alanine	*	*
3	β-Aminoisobutyrate	*	*
4	Alanine	0.970	0.708
5	Alloisoleucine	*	*
6	Arginine	0.937	0.978
7	Asparagine	0.706	0.766
8	Aspartate	0.864	0.865
9	Citrulline	0.998	0.818
10	Cystathionine	*	*
11	Cystine	**	**
12	Ethanolamine	*	*
13	Glutamate	0.881	1.016
14	Glutamine	0.799	1.013
15	Glycine	0.953	0.810
16	Histidine	0.89	0.835
17	Homocitrulline	**	**
18	Homocystine	**	**
19	Hydroxyproline	0.694	0.677
20	Isoleucine	0.987	0.808
21	Leucine	0.963	1.207
22	Lysine	0.953	1.046
23	Methionine	0.998	0.871
24	Ornithine	0.976	1.024
25	Phenylalanine	0.999	1.028
26	Proline	0.958	0.823
27	Sarcosine	*	*
28	Serine	0.766	0.57
29	Taurine	0.924	0.897
30	Threonine	0.853	0.862
31	Tryptophan	0.856	0.753
32	Tyrosine	0.988	0.898
33	Valine	0.981	0.822

- IEC versus LC–MS/MS
  - Plasma samples

***J. Chromatogr. B* (2014) 944: 166– 174**

Good correlation with IEC confirms previously used normal ranges

- Relevant for analytes closely monitored in patients with known IEM

Mass spectrometry can be successfully used for the diagnosis and follow-up of IEM

IEM	Matrix	Amino acid	Concentration (age-related normal range)
MSUD	Plasma	Leu	491 µmol/L (61,0–341,0)
		allo-Ile[a]	115 µmol/L
PKU	Plasma	Phe	1,230 µmol/L (23,5–104,0)
Ornithinemia	Plasma	Orn	660 µmol/L (42.8–346.2)
Treated tyrosinemia type I	Urine	Tyr	86,8 mM/M creatinine (2,8–17,9)
NKHG[b]	Urine	Gly	3971 mM/M creatinine (66,0–416,7)
Cystinuria	Urine	Arg	157,6 mM/M creatinine (6,1–61,7)
		Cys	93,1 mM/M creatinine (2,1–9,8)
		Lys	551,0 mM/M creatinine (4,7–105,0)
		Orn	148,8 mM/M creatinine (0,9–5,4)
Cystathioninuria	Urine	Cth	208 mM/M creatinine (0,3–4,4)

[a] allo-Isoleucine

[b] Non ketotic hyperglycinemia

JJIMD Rep. (2014) 13:119-28

In urine co-eluting compounds interfere with quantification by IEC

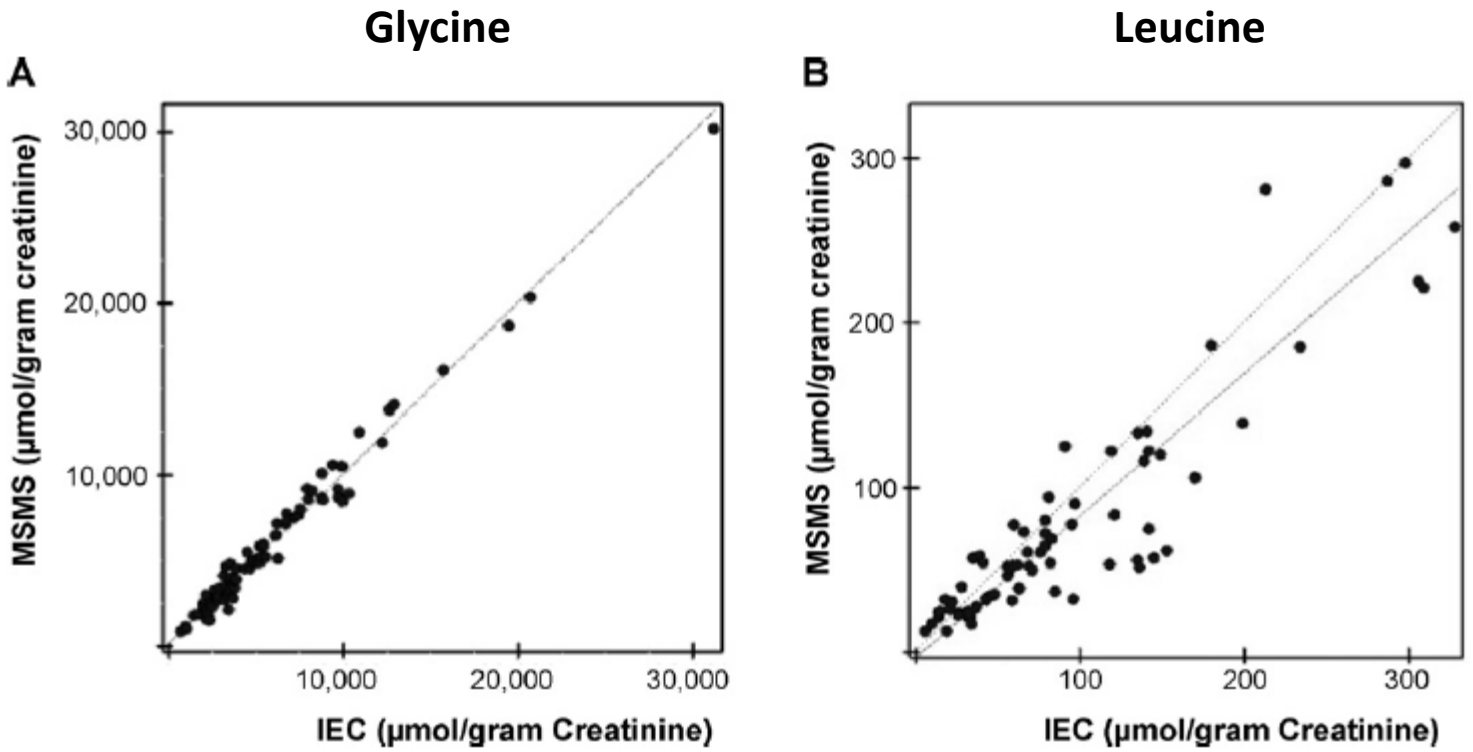
Amino Acid	GC-MS *vs.* Biochrom 20		iTRAQ®-LC-MS/MS *vs.* Biochrom 20	
	R (95% CI)	slope	R (95% CI)	slope
Aad	-	-	-	-
Abu	-	-	-	-
bAib	-	-	-	-
Ala	0.970 (0.959 – 0.978)	0.928	0.944 (0.923 – 0.96)	0.823
Arg	-	-	0.561 (0.437 – 0.663)	0.900
Asn	0.953 (0.935 – 0.966)	0.719	0.940 (0.918 – 0.957)	1.170
Asp	-	-	-	-
Car	-	-	0.801 (0.733 – 0.852)	1.462
Cys-Cys	0.944 (0.922 – 0.959)	0.684	0.811 (0.746 – 0.860)	0.616
EtN	-	-	0.917 (0.886 – 0.939)	0.873
Glu	-	-	-	-
Gln	0.956 (0.94 – 0.968)	1.111	0.938 (0.915 – 0.955)	1.231
Gly	0.980 (0.973 – 0.986)	0.968	0.921 (0.891 – 0.942)	0.730
His	0.969 (0.957 – 0.977)	1.056	0.940 (0.918 – 0.957)	0.799
Ile	0.812 (0.747 – 0.861)	0.812	0.802 (0.736 – 0.854)	0.737
Leu	-	-	-	-
Lys	0.969 (0.957 – 0.978)	0.966	0.951 (0.932 – 0.964)	0.968
M1His	-	-	0.934 (0.909 – 0.952)	0.799
M3His	-	-	0.906 (0.871 – 0.931)	0.753
Orn	-	-	-	-
Phe	0.909 (0.875 – 0.933)	0.778	0.899 (0.862 – 0.926)	1.015
Ser	-	-	0.939 (0.915 – 0.955)	0.856
Tau	-	-	0.885 (0.843 – 0.916)	0.694
Thr	-	-	0.946 (0.925 – 0.961)	1.071
Trp	0.800 (0.733 – 0.851)	0.782	0.760 (0.680 – 0.821)	0.841
Tyr	0.844 (0.788 – 0.885)	0.525	0.807 (0.740 – 0.857)	1.318
Val	0.912 (0.879 – 0.936)	0.995	0.899 (0.862 – 0.926)	0.851

- IEC versus GS-MS and LC–MS/MS using iTRAQ reagents
  - Urine samples

***J. Chromatogr. B* (2009) 877:1838–1846**

Correlation with IEC in urine varies among analytes

- Deming regression model



J. Chromatogr. B (2011) 879:2695– 2703

Analytes difficult to detect by IEC

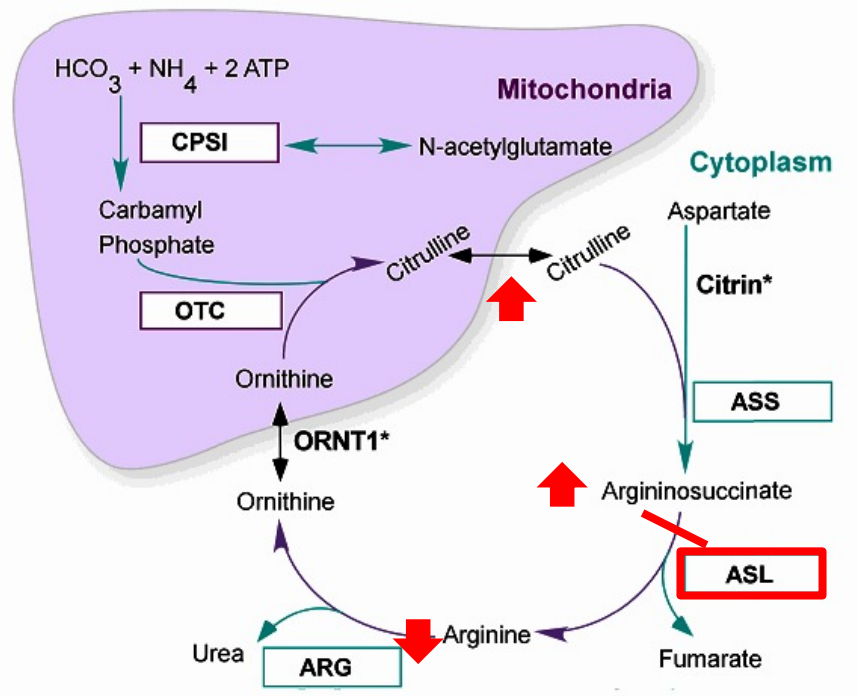
- Homocitrulline
  - Accumulates in patients with defects in the mitochondrial ornithine transporter (Hyperornithinemia-Hyperammonemia-Homocitrullinuria Syndrome)
  - Homocitrulline co-elutes with methionine on a standard IEC chromatogram

Analytes difficult to detect by IEC

- Argininosuccinic acid
  - Precursor to fumarate in the urea cycle, accumulates in patients with argininosuccinate lyase deficiency
  - ASA co-elutes with leucine on a standard IEC chromatogram
- Alloisoleucine
  - Branched-chain amino acid, accumulates in patients with maple syrup urine disease
  - Alloisoleucine co-elutes with cystathionine on a standard IEC chromatogram
- Tryptophan
  - Monitored in Glutaric acidemia type I patients on therapy
  - Tryptophan co-elutes with Histidine on a standard IEC chromatogram

Mass spectrometry may aid the diagnostic process in difficult cases

- Argininosuccinic aciduria
  - Argininosuccinate lyase deficiency (ASL)
  - Converts argininosuccinic acid to arginine & fumarate



Urea Cycle Disorders Overview, GeneReviews

Clinical Presentation

- Neonatal hyperammonemic coma
- Late onset hyperammonemia ± neuropsychiatric disease
- Fragile hair (trichorrhexis nodosa)
- Liver disease with chronic cirrhosis in many cases

Argininosuccinic aciduria Laboratory Findings

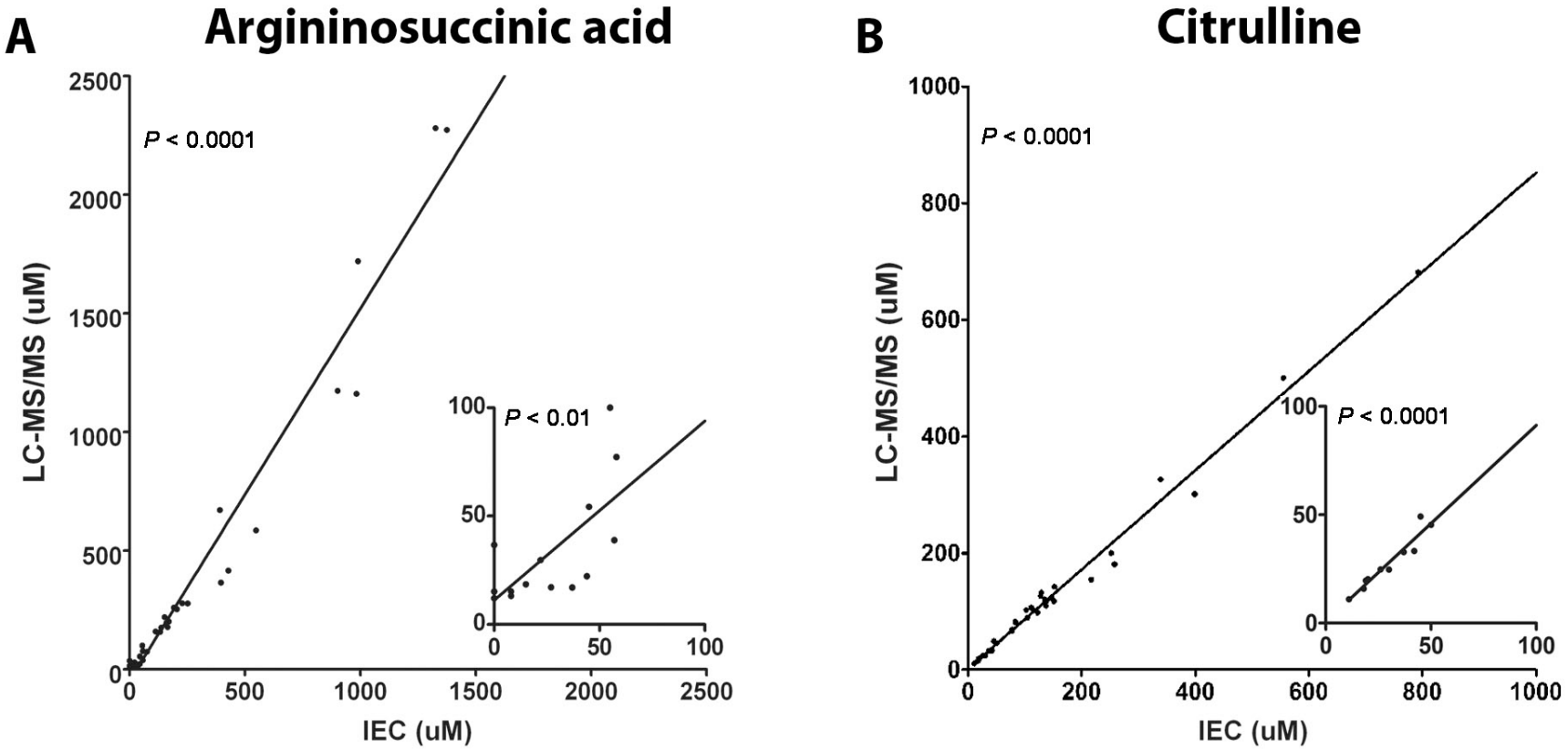
- **Markedly elevated argininosuccinic acid (ASA) in plasma and urine**
- Elevated plasma citrulline
  - Differential diagnosis: Citrullinemia (Type I&II)
- Elevated glutamine (hyperammonemia)
- Low plasma arginine
- Reduced enzyme activity
  - Measured in fibroblasts

Argininosuccinic acid is the key analyte

- Argininosuccinic acid exists in two forms: free acid (usually most abundant) and anhydride
- The argininosuccinic acid-related compounds (free and anhydrides compounds) co-elute with other amino acids by Ion Exchange Chromatography
  - ASA concentration in plasma is lower than in urine. In some patients, plasma ASA can be missed by IEC

LC-MS/MS detects very low ASA amounts

- LC-MS/MS can detect and quantify argininosuccinic acid even when IEC cannot



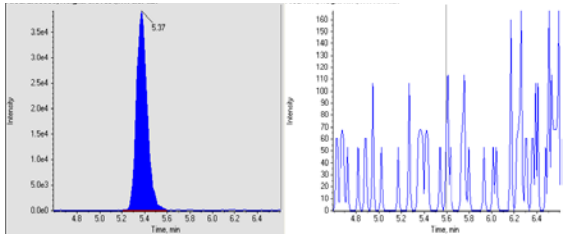
Clinica Chimica Acta (2015) 442:73–74

Argininosuccinic aciduria case study: 6 years old boy

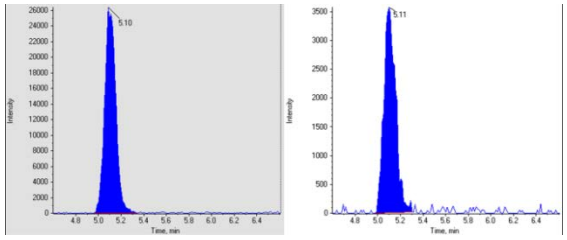
- Developmental delay
- High functioning autism
- Mild hyperammonia
- Normal liver function

Mild Clinical presentation

ASA IS | ASA



Normal plasma profile:
No ASA detected



ASA patient:
ASA = 13.4 µmol/L

- DX confirmed by molecular testing and absence of enzymatic activity
- **ASA not detected by IEC**

Conclusions

- The clinical presentation of metabolic disorders may be non-specific and similar to more common conditions
- The initial work-up for metabolic disorders includes quantitative analysis of amino acids
- Ion Exchange Chromatography is the gold standard; however, methods have been developed that utilize mass spectrometry to increase specificity and throughput

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