



RECOMMENDATIONS TO IMPROVE THE QUALITY OF DIAGNOSTIC QUANTITATIVE ANALYSIS OF AMINO ACIDS IN PLASMA AND URINE USING CATION-EXCHANGE LIQUID CHROMATOGRAPHY WITH POST COLUMN NINHYDRIN REACTION AND DETECTION

This Standard Operating Procedure was developed as part of the EU BIOMED 2-project, BMH4-98-3404 led by Baudouin François MD, Belgium as part of the sub-project SP4 "Calibration standard for amino acids"

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(B. Fowler, Chairman of ERNDIM, May 2002)

GENERAL

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|-----------------------------|---|
| Laboratory | Room should be free of ammonia vapour. Nitrogen pressure facility and fume hood is available. |
| Method / instruments | Automated cation-exchange column liquid chromatography using lithium buffers with post column ninhydrin reaction and dual wavelength detection. High standard amino acid analyser, e.g. Beckman, Pharmacia Alpha Plus, Biochrom Pharmacia or Synkam Analytica. |
| Linearity | At least 500 $\mu\text{mol/L}$ at $\lambda = 570$ nm. At least 1000 $\mu\text{mol/L}$ at $\lambda = 440$ nm. |
| Sensitivity | Lower detection limit is approx. 5 - 10 $\mu\text{mol/L}$ and depending on the individual amino acid. |
| Sample volume | At least 200 μL , preferably 500 μL . |

SAMPLES

A. Collection, initial preparation, storage

Plasma

Blood (1 mL) collected by venipuncture in an lithium-heparin coated tube, after an overnight fasting is strongly preferred.
In case of capillary blood, clean and disinfect skin thoroughly before taking the blood sample, to avoid contamination with amino acids from the skin surface.
Centrifuge (4°C, 15 min 2000 g) and deproteinize within 30 min. after collection (see below).

In case sulphur containing amino acids need not be quantified, deproteinization can be postponed until analysis.

If the sample is not analysed the same day, freeze and store the plasma frozen at -20 °C for max. 2 months or -80 °C for longer period.

Refuse analysis of haemolytic samples.

Other body fluids, e.g. CSF and amniotic fluids need to be collected in tubes without any additional chemicals (e.g. heparin or EDTA) or preservatives.

Urine

A 24-hour urine collection is preferred. Alternatively an overnight collection can be sufficient for diagnostic purposes. No preservatives or other compounds are added.

During the collection period the urine aliquots are kept cold in a refrigerator (4°C) and after completion the urine is sent to the laboratory in a well-isolated package and stored in the refrigerator for max. one week at 4°C until analysis or stored frozen at -20°C when analysis is carried out in more than one week but within 2 months. For longer period store at -80 °C.

Dipstick tests for nitrite and pH should be carried out directly after receipt of the urine in order to check for bacterial contamination. In addition qualitative tests for glucose, reducing substances, sulphite and ketone bodies should be done. Refuse analysis of severely bacterially contaminated samples (pH>7 or nitrite is positive).

In case of thawing urine samples that have been stored frozen, the urine is mixed carefully and thoroughly. 10 mL of the mixed urine is transferred to an uncoated tube and centrifuged (3 min. 10000 g) or filtered (0.2 µm filter). In the supernatant the concentration of creatinine is measured.

Creatinine concentration should be measured using a certified method to enable calculation of creatinine-related excretion values for the amino acids, which can be compared with (age-related) reference values.

If the concentration of creatinine in urine is elevated (>3 mM) the urine sample should be diluted with distilled water before analysis. When the creatinine concentration is 0 - 3 mM no dilution is needed; 3 - 8 mM dilute 2x (= 1 + 1) and if creatinine is > 8 mM dilute 4 x (= 1 +3)..

Before analysis of amino acids the sample should be deproteinized (see below).

B. Deproteinization / preparation for analysis

Plasma

Add 1 volume of a 35 % (w/v) sulfosalicylic acid (SSA) solution containing internal standard to 10 volumes of centrifuged plasma.

Mix thoroughly and incubate 20 min. in refrigerator (4°C), centrifuge at 4°C, 11000g for 10 min. and filter the supernatant through a 0.2 µm filter using a syringe.

Mix the filtered supernatant with an equal volume of sample dilution buffer pH 2.2 (e.g. Lithium-S, Beckman). Use immediately or store in refrigerator(4°C) for max. 3 days.

Urine

Same procedure as for plasma, only a 15 % (w/v) SSA solution is used instead of 35 %.

C. Control samples

Pooled and for some amino acids enriched plasma and urine samples are prepared according to the same procedures as for patient samples (but not deproteinized for storage) and included in each series of diagnostic analyses. The control material can best be stored frozen at -20°C in ready for use aliquots.

REAGENTS / STANDARDS

Before use every reagent and standard should be checked visually for contamination.

Ninhydrin reagent

Preparation / precautions

Prepare reagent exactly according to the manufacturer's instructions. Ninhydrin concentration and specific additions of e.g. Brij-35TM can be important for optimal performance of the reagent. During preparation continuous priming of the solution with nitrogen is also important. For stabilisation of the reagent it should not be used until 24 hours after preparation. Earlier use of the reagent can yield insufficient sensitivity for some amino acids, in particular sarcosine and β -amino isobutyric acid.

Since March 2000 a ninhydrin reagent available as solution can be obtained from Pharmacia. Therefore, the well-known but time-consuming preparation of the ninhydrin reagent should not be used any longer. Further information will be available at the manufacturer's instructions.

Nevertheless, safety precautions are important because of the toxicity of the constituents of the reagent. Wear a safety coat, use gloves, safety glasses and fume hood, in case the ninhydrin reagent is self-prepared.

When ninhydrin reagent is spilled on the skin it can be removed with a solution of sodium disulphite 10%; afterwards skin and hands should be washed carefully.

Storage and stability

Store the home-made ninhydrin reagent in a refrigerator (at 4°C) under nitrogen for max. 1 month. Usage after this period can produce noisy baselines and lower sensitivity.

Buffers

Lithium citrate buffers for sample dilution and gradient elution are prepared according to the manufacturer's instructions and stored in a refrigerator (at 4°C) until the stated expiry date.

Safety rules:

Be aware of the safety rules, especially with respect to usage and storage of e.g. phenol, methanol, HCl, Li-OH, Li-citrate reagents, hydrindantin and ninhydrin reagents.

In general: wear a safety coat, use gloves, use safety glasses and prepare the buffers in a fume hood.

Standards

Internal standards

Preferably two different compounds eluting in the most important parts of the chromatogram, e.g.

S-2-aminoethyl-1-cysteine (AEC) and glucosaminic acid (GLUA) can be quite suitable, depending on the analyser system. These internal standards should be added in a fixed amount to all samples, including calibrators and controls.

Internal standard solutions are stored in a 1 % (w/v) SSA solution in a refrigerator (4°C). Stability is max. 2 years under these conditions.

Calibrators_

Aqueous solutions of physiological amino acids in dilution buffer pH 2.2, with amino acids on reasonable levels (typically 250 µmol/L) are used to calibrate each series of analyses. For special programs to analyse single or specific amino acids comparable solutions with relevant levels are used.

It is also useful to add some unusually present amino acids e.g. pipercolic acid (PIP), alloisoleucine (AILE), cystathionine (CYSTA) en homocystine (HCY2). Because of instability of glutamine (GLN) it has to be added just before use. I The calibrator is stored at -80°C, max. 1 year.

External standards

At regular times external standard solutions comparable with the calibrator solutions, but of independent origin (different manufacturer or self-made) are used as a control of calibration.

ANALYSIS

Preparations

All reagents and chemicals are used at room temperature.
Home-made ninhydrin reagents has been prepared at least 24 hours before use (during storage no oxidation is allowed).
Collect: samples (thawed), calibrators, standards, controls and SSA.
Deproteinize samples as described in Samples part B

Analysis

The elution program used for diagnostic amino acid analysis should distinguish qualitatively and quantitatively between the amino acids mentioned in enclosure 1.

In analytical validation it is essential to note:

1. Inspect the chromatogram visually (base line, peak areas, elution pattern);
2. Check internal standards with respect to retention time and peak areas.
3. Verify positions and areas of the various amino acids; estimation of every individual amino acid should take place very careful.

If step 1, 2 or 3 in this process gives rise to any doubt about the reliability of the analysis, the analytical problem needs to be identified and solved first. In addition the complete analytical series has to be repeated.

If identification of individual amino acids is compromised by deviant retention behaviour the elution program should be readjusted.

When problems remain you might contact the Scientific Advisor of the ERNDIM QC scheme for amino acids (see www.ERNDIMQA.NL).

Please mention the analyser used, the elution program used, set up of the analysing program, internal standards used and what is found and the amino acids which could not be identified.

QUALITY CONTROL

Internal QC / analytical validation

Recoveries, detection limits and linearity of all amino acids have to be established by analysing plasma and urine samples before and after enrichment (standard addition method) with reference compounds to define these parameters in the relevant biological matrix.

Exact retention times and response factors for each amino acid have to be determined at two wavelengths ($\lambda = 570$ nm and $\lambda = 440$ nm). Signal ratios at these wavelengths have to be determined for proper identification of the individual amino acids and detection of co-eluting interferences.

Control plasma or urine samples are analysed in each series of plasma or urine amino acid analyses to control the inter-assay reproducibility.

External standard solutions are analysed at regular intervals, e.g. monthly or weekly, depending on the analysis frequency.

Internal standards are added to all samples including calibrators, external standards and control samples.

All values obtained for standards and control samples are registered and compared with the calculated respectively expected values and in case of deviations of > 2 s.d. the series is repeated.

A reagent blank (= only loading buffer) is run occasionally to check for any contaminants in the reagents.

Instrument performance is checked by observing the baseline and noise level, and looking at the amino acids in the lowest concentrations (signal to noise ratio).

In general:

Analytical detection of each amino acid depends on:

1. Quality and condition of the analytical column.
2. Temperature during reaction with ninhydrin reagent;
3. Response factor in the linear area (is different for each amino acid);
4. Performance of the photometer (detector).

If any changes occur with respect to step 1 (new column), 2 (e.g. new ninhydrin reagent), 3 or 4 (e. g. new lamp) changes may occur in the linear part of the calibration curve.

With every change in the procedure mentioned it is required to check for every amino acid whether the response factor in the linear part of the calibration curve is still correct or needs to be adjusted.

External QC

Participation in an external QA scheme like the ERNDIM (European Research Network for evaluation and improvement of screening, diagnosis and treatment of inherited Disorders of Metabolism) scheme "Quality Assurance Program for Quantitative Amino acid Analyses" organised by SKZL (Stichting Kwaliteitsbewaking Ziekenhuis Laboratoria) at Nijmegen (NL) on behalf of ERNDIM is strongly recommended.

For some amino acids the QA scheme of "Special assays in serum and urine" also organised by SKZL on behalf of ERNDIM is available.

LIMITATIONS AND PITFALLS

Instability of amino acids

Glutamine and asparagine gradually disappear during storage, even in frozen samples, while glutamic and aspartic acids increase concomitantly. Phosphoethanolamine decomposes to ethanolamine (and phosphate). Tryptophan, GABA and taurine usually increase. Slight increases of methionine, tyrosine, ornithine, lysine and histidine also often occur.

Exogenous artefacts

Anticoagulants can contain interfering constituents, e.g. blood collection tubes containing sodium bisulphate in addition to heparin can yield a peak of sulphocysteine, falsely suggesting sulphite oxidase deficiency. EDTA can produce several ninhydrin-positive artefacts.

Hemolysis and or contamination of the plasma with blood cells can cause a number of artefacts, a.o. decrease of arginine with simultaneous increase of ornithine and in addition of aspartic and glutamic acids, glycine and glutathione (red cells). Leukocytes and platelets can contribute taurine.

Bacterial contamination enhances the conversion of glutamine and asparagine to glutamic and aspartic acids, and particularly in urine decreases of glycine, alanine, proline and many other amino acids can be observed, although even increase of glycine is possible as a result of bacterial decomposition of hippuric acid. Cystathionine can be converted to homocystine, mimicking homocystinuria.

Fecal contamination of urine can cause serious increases of mainly proline, glutamic acid and branched-chain amino acids

Tryptophan can be lost variably due to deproteinization, while delayed deproteinization causes losses of disulfide-containing amino acids, mainly cystine and homocystine.

The same effect occurs during clotting, and therefore makes serum unsuitable as a sample for these amino acids.

Also in protein containing urines these losses occur.

Medication and dietary artefacts are almost innumerable.

Only some examples are referred, such as erroneously high tyrosine peaks in case of penicillin antibiotics, phenylalanine in paracetamol medication; glycine and alanine in use of valproate; GABA, β -alanine and β -amino-isobutyric acid in γ -vinyl-GABA (vigabatrinTM).

Hyperalimentation, particularly with high protein content can lead to hyperaminoacidemia/-uria.

Consumption of meat and especially poultry leads to increased excretion of β -alanine-containing dipeptides (carnosine, anserine), 1-methylhistidine and β -alanine.

Collagen-rich diets can cause increased excretion of hydroxyproline and hydroxylysine-containing dipeptides.

LITERATURE

1. Spackman D.H., Stein W.H., Moore S., *Analyt.Chem.* 30, 1185-1190 (1958).
2. *Handbook Amino Acid Analysis Theory & Laboratory Techniques* (1987) Pharmacia.
3. Deyl Z., Hyánek J., Horáková M., *Journal of Chromatography*, 379 (1986) 177-250.
4. Bremer H.J., Duran M. et al, *Disturbances of Amino Acid Metabolism: Clinical Chemistry and Diagnosis* (1981).
5. Scriver, C.R. et al., *The metabolic inherited disease.*

APPENDIX

1. Abbreviations of the amino acids used.

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Appendix

Abbreviations used

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| phosphoserine | PPS |
| sulfocysteine | SULFO |
| taurine | TAU |
| phosphoethanolamine | PEA |
| aspartic acid | ASP |
| hydroxyproline | HYP |
| threonine | THR |
| serine | SER |
| asparagine | ASN |
| glutamic acid | GLU |
| glutamine | GLN |
| sarcosine | SAR |
| α -aminoadipic acid | AAA |
| proline | PRO |
| glycine | GLY |
| alanine | ALA |
| citrulline | CIT |
| α -aminobutyric acid | ABU |
| valine | VAL |
| cystine | CYS2 |
| methionine | MET |
| homocitrulline | HCI |
| cystathionine | CYSTA |
| isoleucine | ILE |
| leucine | LEU |
| tyrosine | TYR |
| β -alanine | BALA |
| phenylalanine | PHE |
| β -aminoisobutyric acid | BAIB |
| homocystine | HCY2 |
| γ -aminobutyric acid | GABA |
| ethanolamine | ETN |
| 3-hydroxykynurenine | HYK |
| kynurenine | KYN |
| hydroxylysine | HYL |
| ornithine | ORN |
| lysine | LYS |
| 1-methylhistidine | 1MHIS |
| histidine | HIS |
| tryptophan | TRP |
| 3-methylhistidine | 3MHIS |
| anserine | ANS |
| carnosine | CAR |
| arginine | ARG |
| homo-arginine | HAR |
| allo-isoleucine | AILE |
| argininosuccinic acid | ASA |

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