ADVANCES IN PHYSIOLOGICAL AMINO ACID ANALYSIS

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ABSTRACT

Objective: Amino acids analysis (AAA) is used as a tool by clinicians to research various medical conditions and the treatment of those conditions. This analysis is complex both because the number of required amino acids is large and the samples can contain a large and unpredictable array of interferences. The existing AAA methods have relatively long run times, limited sensitivity, high cost per sample, and can be unreliable. To develop a new analytical solution, all of these concerns must be addressed within a framework that yields an accepted "correct" answer in a robust and reliable method. A complete system approach was chosen. The solution includes a well-characterized amino acid derivatization chemistry, a tested UltraPerfomance LC® method for analysis of the derivatized amino acids, amino acid standards, chromatographic columns, eluents and software for both data acquisition and customized reporting.

Methods: The amino acids are derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). Following the pre-column derivatization of the analytes, separation and detection are achieved with a reversed-phase UPLC® column and TUV detector, respectively. The analysis is complete within approximately 45 minutes and allows for the identification and quantification of 42 amino acids and related compounds. These samples are automatically analyzed with assured performance methods and reports are generated using pre-defined software templates.

Results: The quality and robustness of the analysis was studied to ensure accurate identification and quantification of all the amino acids screened. The amino acid standard includes the common acidics, neutrals and basic amino acids mixtures, freshly supplemented with glutamine and allo-isoleucine. Norvaline is included as an internal standard. The amino acids are identified by retention time relative to a standard. Retention time precision for a standard is typically 0.2% RSD between runs. Quantification by peak area, with comparison to a standard, shows between run precision of typically 1.3% RSD. The limit of detection for the method is observed to be 50 femtomole on column; this corresponds to 0.5 µmol/L of each amino acid in the starting sample. Linearity from 100 fmoles to 50 pmoles/µL is shown with <20% deviation from calculated values for the lower limit of 100 femtomoles on column, or 1 µmol/L of amino acid in sample. The method has been tested with plasma and urine samples. The method is compatible with several deproteinization procedures, including sulfosalicyclic acid (SSA) and ultrafiltration. The precision studies described involve plasma samples deproteinized with SSA, and containing norvaline as an internal standard. A standard derivatization uses the equivalent of 10-20µL of plasma or urine and can be scaled down where required. For plasma samples, retention time precision was typically 0.2% RSD between runs. The area precision of the deproteinized samples was typically 2% RSD between sample. These samples have also been compared to standard methods.

INTRODUCTION

For several decades, amino acid analysis has been used in the study of a number of physiological processes. A correlation with the metabolic disturbances and amino acid levels in plasma and/or urine has been shown. Along with the ever increasing observations and discoveries of these conditions, has come the need to identify and quantify amino acids at levels of as low as 1 µmol/L. With these requirements in mind, the MassTrak™ AAA Solution has been developed to provide a full profile physiological amino acid analysis for research use only. It is a turnkey method that is robust and reliable with higher throughput than is customary. The MassTrak AAA Solution is comprised of pre column derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. The resulting dervatized amino acids are identified and quantified using an ACQUITY UPLC® with a TUV detector.

METHODS

DERIVATIZATION

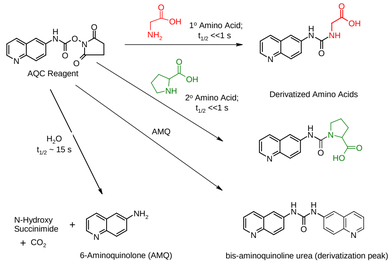

Figure 1. Reaction of AQC reagent with amino acids. The 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) reagent reacts with both primary and secondary amines. Excess reagent reacts with water to form 6-aminoquinoline (AMQ). Subsequently, AMQ can react with excess AQC reagent to form a bis urea. Both of these side products do not interfere with the identification of any of the amino acids. The derivatives are stable for days, permitting batch-wise processing.

CHROMATOGRAPHIC CONDITIONS

Column: MassTrak™ AAA 2.1 x 150 mm, 1.7µm
Mobile Phases: MassTrak™ Eluent A and Eluent B
Flow Rate: 0.4 mL/min
Injection Volume: 1.0 µL
Gradient: MassTrak AAA Standard Gradient
Column Temp: 43 °C
Detection: UV @ 260 nm
Instrument: ACQUITY UPLC with TUV

SAMPLE PREPARATION

The MassTrak AAA Solution is utilized for the analysis of physiological amino acids in both urine and plasma. Plasma is deproteinzed with an equal volume of 10% sulfosalicylic acid (prior to derivatization. Urine samples do not require deproteinization. For derivatization of plasma, 20µL of supernatant, 60µL of borate buffer, and 20 µL of reagent are mixed. For analysis of urine samples, 5µL of urine, 75µL of borate buffer, and 20 µL of reagent are combined. The 1 µL injection volume is equivalent to 100 nL of plasma or 50 nL of urine.

RESULTS AND DISCUSSION

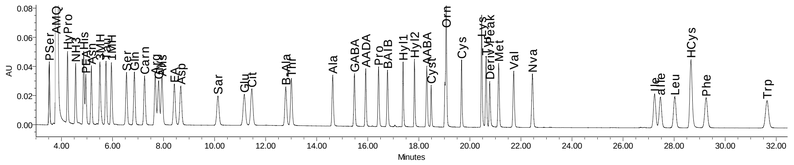

Figure 2. Chromatogram of physiological amino acids (250µmol/L) with MassTrak AAA Solution. The amino acids include glutamine (Gln), allo-isoleucine (aIle), as well those found in the Acidics and Neutral and Basics amino acid solutions. Norvaline (Nva) is used as the internal standard.

The standard chromatogram shown in Fig. 2 is evaluated for its suitability based on peak identification and reliability of quantification. Identification is based on retention time and the reproducibility of retention time is summarized in Table 1. Interrun variability of retention time is much less than the retention difference between adjacent peaks. Identification is, therefore, not ambiguous.
Reliable quantification is dependent on reproducibility, sensitivity and linearity. Interrun quantitative variability averages less than 1% with internal standard as shown in Table 1.
The limit of detection for the method has been found to be 0.5 µmol/L (Figure 3). The limit of quantification is 1 µmol/L as determined in the linearity experiments.
Each individual amino acid exhibits a linear response from 1 µmol/L to 10 mmol/L with a R^2 of >0.995. As an example, the results are shown for Phe in Figure 7. For a complete mixture of the 42 amino acids linearity has been demonstrated from 1µmol/L to 500µmol/L with a R^2 of greater than 0.995.
These ranges exceed the commonly observed levels of the amino acids in physiological samples.

Amino Acid	%CV R_T	%CV Area Ratio	Amino Acid	%CV R_T	%CV Area Ratio
PSer	0.18	1.30	GABA	0.03	1.15
HyPro	0.15	0.41	AADA	0.03	1.00
His	0.14	0.42	BAIB	0.02	0.70
PEA	0.14	1.34	Pro	0.02	0.92
Asn	0.14	0.62	Hyl1	0.01	0.71
3MH	0.13	0.46	Hyl2	0.01	0.74
Tau	0.14	0.61	AABA	0.02	0.85
1MH	0.12	0.56	Cyst	0.01	0.59
Ser	0.13	0.97	Orn	0.01	0.91
Gln	0.12	0.66	Cys	0.01	0.58
Carn	0.11	0.61	Lys	0.01	0.89
Arg	0.12	0.56	Tyr	0.02	0.41
Gly	0.11	0.55	Met	0.01	0.58
Ans	0.10	0.56	Val	0.01	0.76
EA	0.10	1.60	Nva	0.01	0.73
Asp	0.13	1.86	Ile	0.02	0.80
Sar	0.09	0.97	aIle	0.02	0.67
Glu	0.11	0.97	Leu	0.02	0.69
Cit	0.10	0.65	HCys	0.02	0.71
B-Ala	0.06	1.66	Phe	0.02	0.49
Thr	0.05	1.59	Trp	0.03	0.51
Ala	0.03	0.93			

Table 1. Interrun precision and accuracy. MassTrak AAA Standard, 250 µmol/L duplicate derivatizations, triplicate injections. Six injections total.

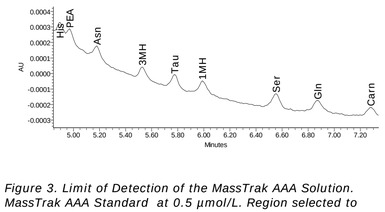

Figure 3. Limit of Detection of the MassTrak AAA Solution. MassTrak AAA Standard at 0.5 µmol/L. Region selected to show details.

X Value (µmol/L)	% Deviation
1.22	15.20
2.44	5.49
4.88	0.78
9.77	-4.63
19.53	-4.06
39.06	-3.15
78.13	-3.64
156.25	-1.69
312.50	-0.49
625.00	-1.85
1250.00	-0.88
2500.00	-0.59
5000.00	-1.89
10000.00	1.41

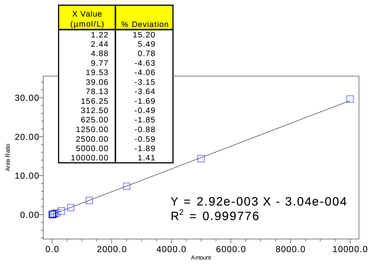

Figure 4. Calibration curve for Phe (1.25 µmol/L to 10mmol/L).

ANALYSIS OF PHYSIOLOGICAL SAMPLES

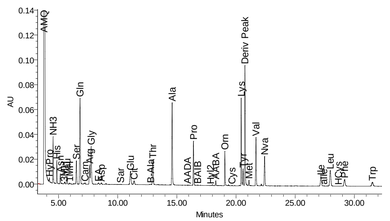

Figure 5. Chromatogram of a pooled human plasma sample.

Amino Acid	%CV R_T	%CV Area Ratio	Amino Acid	%CV R_T	%CV Area Ratio
HyPro	0.11	0.57	Thr	0.09	0.75
His	0.14	0.93	Ala	0.05	0.67
Asn	0.14	0.86	Pro	0.03	0.55
Tau	0.16	0.94	AABA	0.02	0.85
1MH	0.16	1.58	Orn	0.02	0.72
Ser	0.16	1.51	Lys	0.02	1.15
Gln	0.17	0.83	Tyr	0.02	1.29
Carn	0.18	1.17	Met	0.01	1.06
Arg	0.19	0.92	Val	0.01	0.60
Gly	0.16	0.88	Ile	0.02	0.73
EA	0.16	0.60	Leu	0.02	0.71
Asp	0.18	1.53	Phe	0.03	1.09
Glu	0.05	0.76	Trp	0.05	1.49
Cit	0.18	0.79			

Table 2. Interrun precision and accuracy. Pooled human plasma. Triplicate derivatizations, Triplicate injections. Nine injections total.

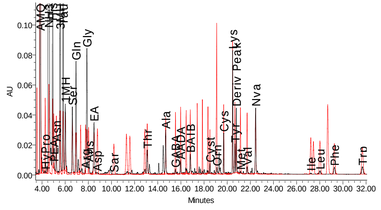

Figure 6. Overlay chromatogram of derivatized human urine sample and MassTrak AAA Standard (250 µmol/L). There is no difference in retention time between the sample and the stan-

COMPATIBILITY WITH MS DETECTION

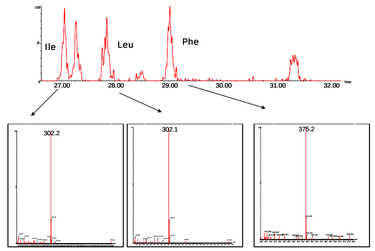

Figure 7. In this assay MS analysis is not required for routine peak identification or quantification. However, unexpected or unidentified peaks may be observed in physiological samples. Identification of these peaks may be facilitated by collecting MS data. The MassTrak AAA method is compatible with electrospray ionization.

CONCLUSION

- **The MassTrak AAA Solution provides a complete turnkey approach to the analysis of physiological amino acids for research use only.**
- **Stable derivatives are formed for both primary and secondary amino acids.**
- **The chromatographic separation provides unambiguous identification of the amino acids.**
- **Reproducibility of quantification is better than 2% CV.**
- **The demonstrated linearity exceeds the levels that are commonly observed in physiological samples.**
- **Analysis requires low microliter volumes of sample.**
- **Chromatographic method is compatible with electrospray MS detection.**
- **The MassTrak AAA Solution is a robust and reliable, high throughput tool for the quantitative analysis of physiological amino acids for research use only.**

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