Rapid and Reproducible Amino Acid Analysis of Physiological Fluids for Clinical Research Using LC/MS/MS with the aTRAQ™ Kit

Fast, simple and cost effective analysis

Many areas of biochemical research and clinical medicine require analysis of amino acids and other amine-containing compounds. Individual amino acid levels are measured to research metabolic disorders and other disease conditions in physiological samples. It was recently identified that the levels of six metabolites significantly increased as prostate cancer progressed from benign to metastatic states[1]. These metabolites, of which four are amino acids (sarcosine, leucine, proline, kynurenine), can therefore potentially serve as markers for prostate cancer progression, illustrating growing areas of interest for research into amino acid quantitation. Samples analyzed for amino acids are plasma, urine, cerebrospinal fluid (CSF), and tissue extracts. Current dedicated amino acid analyzer systems have long run times, are prone to interferences from buffers, matrices, and other co-eluting amino acids, and can be difficult to maintain. An LC/MS/MS method for the analysis of amino acids in physiological fluids using amine-reactive isotope-coded tags (aTRAQ™ reagents) is described. Because only compounds with the same mass require chromatographic separation, the analysis time can be significantly reduced versus traditional methods. The combination of LC/MS/MS with aTRAQ™ reagents also provides better sensitivity (therefore lower detection limits), a wider dynamic range, and the ability to use labeled internal standards for more accurate and robust quantitation.

Key Features of aTRAQ™ Kit

- Ability to analyze 45 amino acids in a single run
- High throughput with an analysis time of 18 minutes per sample
- Single universal protocol for a range of physiological fluids and matrices such as plasma, urine, tissue extracts, culture media, etc.
- Small sample requirement (40 µl)
- Internal standard for each analyte, leading to accurate and precise analyte identification and quantitation
- Wide dynamic range, with LLOQ and ULOQ of <1 µM to >10,000 µM, respectively
- Complete kit that includes the required reagents, buffers, standards, controls, and column
- Ability to add custom amines, amino acids, and small peptides and create corresponding internal standards
- Ability to automate pipetting steps (optional)

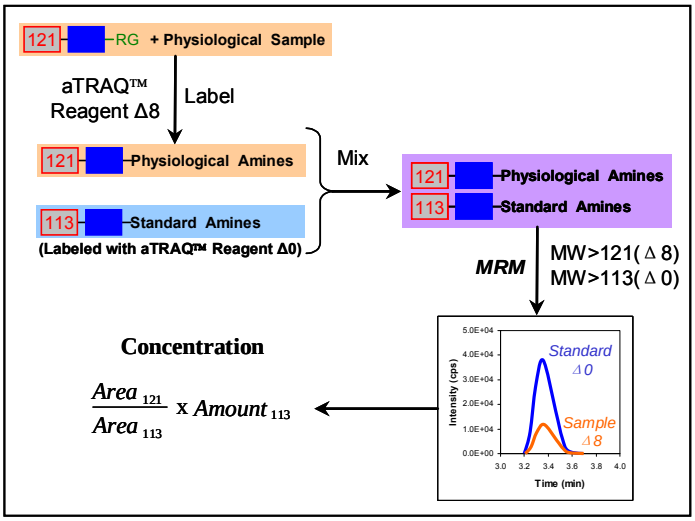

Figure 1: Illustration of aTRAQ™ reagent labeling. The sample is labeled with the Δ8 reagent and the internal standard (IS) is pre-labeled with the Δ0 reagent. The sample and IS are mixed and injected for LC/MS/MS analysis. Quantification of the individual amino acids is calculated using a ratio of the sample to internal standard peak areas. Using an internal standard generates more accurate results.

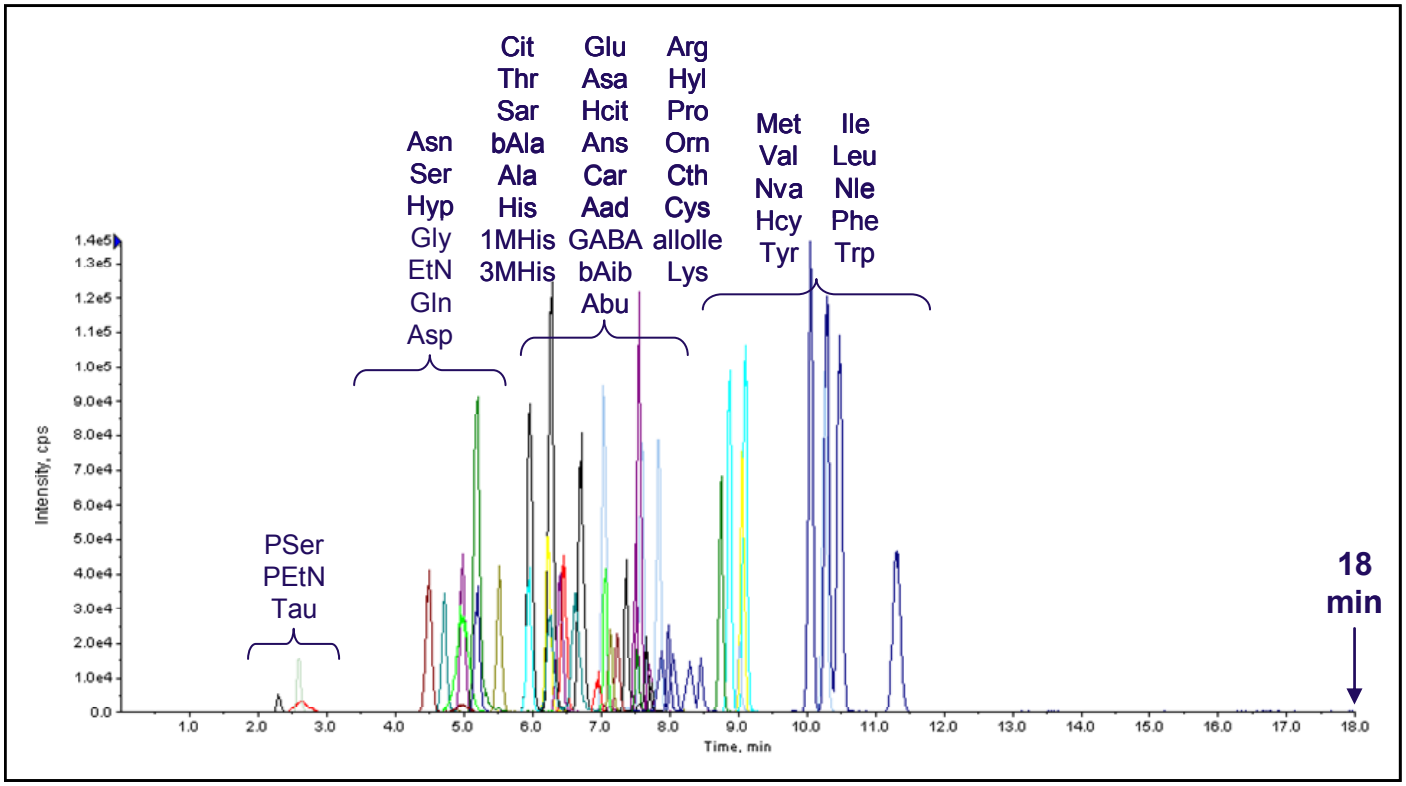

Figure 2: Analyze 45 amino acids. Representative chromatogram showing analysis of 45 amino acids using aTRAQ™ reagents and LC/MS/MS analysis. Total run time is 18 minutes.

Experimental

Analysis was carried out by first precipitating any proteins out of the sample with sulfosalycylic acid, followed by derivatization with the reagents supplied in the aTRAQ™ Kit for Amino Acid Analysis. The samples labeled with aTRAQ™ reagent Δ8 are mixed with the internal standards pre-labeled with aTRAQ™ reagent Δ0. This mixture is then injected for LC/MS/MS analysis using either a triple quadrupole or QTRAP® system operating in MRM mode. The use of the Scheduled MRM™ Algorithm maximizes dwell time while monitoring of large numbers of MRM transitions, resulting in optimum data quality and reproducibility. Quantitation is performed using Analyst® software or Cliquid® software for amino acid analysis. An illustration of analyte labeling and amino acid quantitation is shown in Figure 1. All data shown were acquired using an Agilent 1100 HPLC system interfaced to an AB SCIEX 3200 QTRAP® Mass Spectrometer. Separation was achieved using an Applied Biosystems® AAA C18 Column.

Results and Discussion

A representative chromatogram from analysis of the labeled amino acids in a physiological standard mix is shown in Figure 2. Analytical run time, including column equilibration, takes 18 minutes. Fast separation can be achieve because only isobaric (same mass) amino acids require complete chromatographic resolution, which is accomplished as illustrated in Figure 3. Figure 4 compares results from a plasma sample analyzed using the conventional ninhydrin-based amino acid analyzer with results obtained using aTRAQ™ labeling and LC/MS/MS analysis. The results between the two techniques showed good agreement. Not all amino acids are shown because interference from buffers, matrices, and other co-eluting amino acids limits the number of amino acids reported for the conventional ninhydrin based analyzer.

To test the precision and accuracy of the method, three samples of the same standard mix were labeled and each injected 3 times. The average accuracy was 94% and the precision of the measurement averaged 2.8%. Table 1 lists the precision and accuracy for the analysis of individual amino acids. The lower limit of quantitation (LLOQ) for amino acid analysis using aTRAQ™ reagents was ≤1 µM for all amino acids and the upper limit of quantitation (ULOQ) was ≥10,000 µM, resulting in a linear dynamic range of at least 4 orders of magnitude. Table 2 lists the LLOQ and ULOQ for each amino acid.

Retention time stability is an important part of this method for two reasons: (1) identification and quantitation of compounds that share MRM transitions (Figure 3), and (2) the use of scheduled MRM acquisition.

ALLO-ISOLEUCINE ANALYSIS

Labeled allo-isoleucine does not separate from labeled isoleucine. If allo-isoleucine quantitation is desired, the analysis can be done using an unlabeled sample with unlabeled norleucine as the internal standard. To measure the concentration of allo-isoleucine, the sample is derivatized and the excess aTRAQ™ reagent quenched with hydroxylamine. An aliquot of the unlabeled sample is added to the labeled sample and both the labeled amino acids and unlabeled allo-isoleucine can be analyzed in a single LC/MS/MS run. Figure 5 shows analysis of a plasma sample, demonstrating separation and quantitation of unlabeled allo-isoleucine. Plasma was analyzed alone or spiked with 100 µM allo-isoleucine.

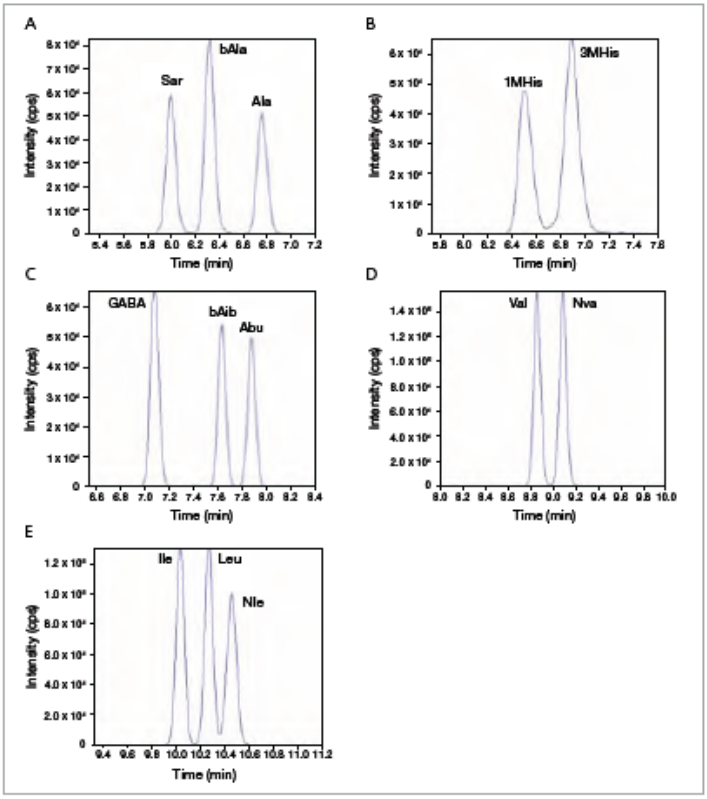

Figure 3: Separation of amino acids with the same mass: a)sarcosine/β-alanine/alanine, b) 1-methyl histidine/3-methyl histidine, c) γ-aminobutyric acid,/β-aminoisobutyric acid/α-aminobutyric acid, d) valine/norvaline, and e) isoleucine/leucine/norleucine).

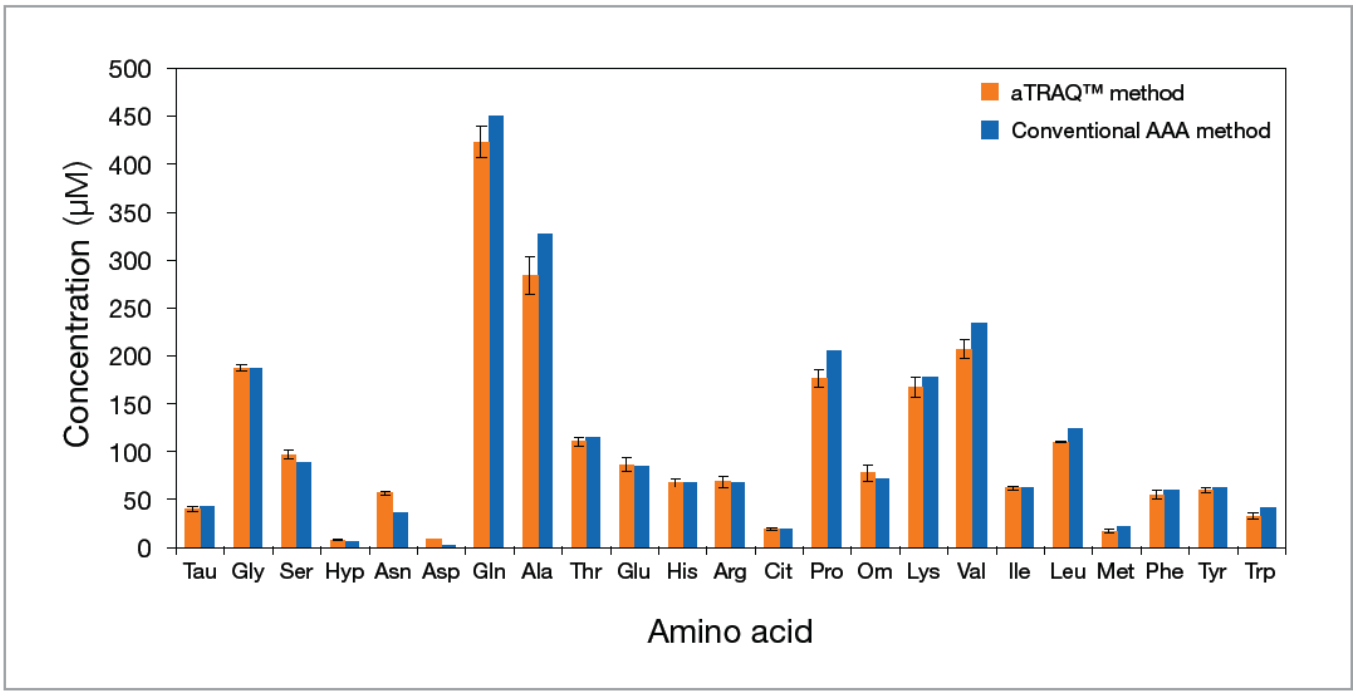

Figure 4. Comparison of results from a plasma sample analyzed using both a conventional ninhydrin based amino acid analyzer and LC/MS/MS analysis using aTRAQ™ reagents. Data from the two methods were in good agreement. Results for all 45 amino acids are not shown because interferences from buffers, matrices, and/or co-eluting amino acids limited the ability of the conventional amino acid analyzer to accurately quantitate some compounds.

Amino acid	Concentration (µM)	Expected Concentration (µM)	Accuracy (%)	Precision (%)
PSer	82.9	95	87%	3.4%
PEtN	88.4	95	93%	2.8%
Tau	84.9	95	89%	2.3%
Asn	92.7	95	98%	4.4%
Ser	87	95	92%	3.0%
Hyp	90.9	95	96%	3.6%
Gly	93.3	95	98%	4.5%
Gln	93.7	95	99%	3.3%
Asp	90.8	95	96%	3.9%
His	94.4	95	99%	4.3%
Thr	89.8	95	95%	1.5%
Cit	82.5	95	87%	3.2%
Sar	104.8	95	110%	3.3%
bAla	101.8	95	107%	3.0%
Ala	83.4	95	88%	3.4%
Glu	90.4	95	95%	2.8%
1MHis	88.9	95	94%	2.3%
3MHis	91	95	96%	2.1%
Asa	90.5	95	95%	3.0%
Car	87	95	92%	2.0%
Ans	86.1	95	91%	2.1%
Hcit	83.3	95	98%	2.8%
Arg	87.6	95	92%	3.1%
Aad	88.4	95	93%	1.9%
GABA	88.2	95	93%	4.0%
bAib	85.3	95	90%	2.1%
Abu	87.8	95	92%	2.1%
Hyl	87.1	95	92%	3.2%
Pro	89.3	95	94%	2.5%
Om	86.9	95	91%	3.0%
Cys	47.3	50	95%	2.9%
Lys	86.6	95	91%	2.8%
Val	86.4	95	91%	1.9%
Nva	99.2	95	104%	2.0%
Tyr	86.9	95	91%	1.9%
Hcy	85.3	95	90%	3.1%
Lle	89.8	95	95%	2.9%
Leu	86.9	95	91%	2.3%
Nle	91.8	95	97%	4.3%
Phe	90.4	95	95%	2.0%
Trp	88.4	95	93%	1.6%
		average	94%	2.8%

Table 1: Precision and accuracy for amino acid analysis. Three aliquots of a standard mixture were labeled and each analyzed in triplicate. The average accuracy was 94% and average precision was 2.8%.

Amino acid	LLOQ (µM)	ULOQ (µM)	Amino acid	LLOQ (µM)	ULOQ (µM)
1MHis	0.2	>10,000	Hcy	0.5	>10,000
3MHis	0.2	>10,000	His	0.5	>10,000
Aad	0.2	>10,000	Hyl	0.5	>10,000
Abu	0.2	>10,000	Hyp	0.2	>10,000
Ala	0.2	>10,000	Ile	0.5	>10,000
Ans	0.5	>10,000	Leu	0.5	>10,000
Arg	0.5	>10,000	Lys	0.5	>10,000
Asa	1	>10,000	Met	0.1	>10,000
Asn	0.5	>10,000	Nle	0.2	>10,000
Asp	0.1	>10,000	Nva	0.2	>10,000
bAib	0.2	>10,000	Orn	0.5	>10,000
bAla	0.5	>10,000	PEtN	0.5	>10,000
Car	0.5	>10,000	Phe	0.2	>10,000
Cit	0.5	>10,000	Pro	0.1	>10,000
Cth	0.5	>10,000	PSer	0.5	>10,000
Cys	1	>10,000	Sar	0.2	>10,000
EtN	0.5	>10,000	Ser	0.5	>10,000
GABA	0.05	>10,000	Tau	0.5	>10,000
Gln	0.5	>10,000	Thr	0.2	>10,000
Glu	0.5	>10,000	Trp	0.1	>10,000
Gly	1	>10,000	Tyr	0.5	>10,000
Hcit	0.2	>10,000	Val	0.2	>10,000

Table 2. Lower (LLOQ) and upper (ULOQ) limits of quantitation for each amino acid. Linearity spanned at least four orders of magnitude for each amino acid.

Summary

The LC/MS/MS method for amino acid analysis presented here reduces run times, therefore increasing throughput, versus conventional amino acid analytical techniques. The use of an internal standard for each analyte results in more accurate and robust quantitation, yielding increased confidence in results. Furthermore, this method also provides low detection limits, a wide linear dynamic range, and the ability to resolve key amino acids. The combination of aTRAQ™ Kits and LC/ MS/MS analysis enables a fast and accurate quantitative analysis of amino acids in physiological fluids.

References

1. Sreekumar, A., et. al., “Metabolic profiles delineate potential role for sarcosine in prostate cancer progression,” Nature, 457(12), 2009, 910-915.

Literature code: 050010-01

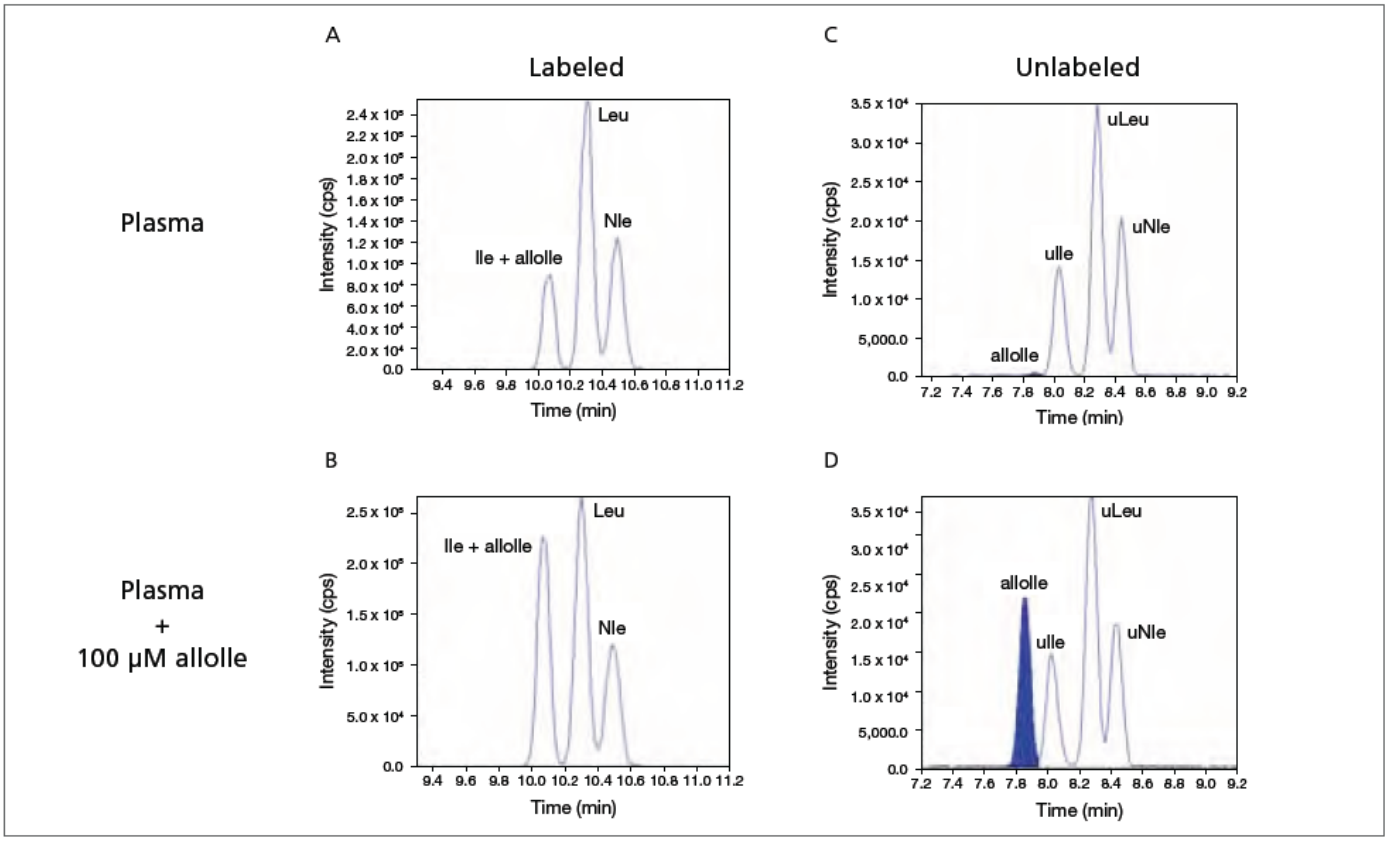

Figure 5. Separation and quantification of allo-isoleucine. allo-Isoleucine and isoleucine co-elute when labeled with aTRAQ™ reagents. Therefore, alloisoleucine can only be separated from isoleucine as an unlabeled species. To quantify allo-isoleucine, a sample is derivatized and the excess aTRAQ reagent quenched with hydroxylamine. An aliquot of the unlabeled sample is added to the labeled sample and this mixture is analyzed in a single LC/MS/MS run. Plasma was analyzed alone or spiked with 100 µM allo-isoleucine. Panels A and B show that the labeled isoleucine and allo-isoleucine co-elute. Panels C and D show the separation of unlabeled isoleucine and allo-isoleucine

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