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Rapid amino acid quantitation with pre-column derivatization; ultra-performance reverse phase liquid chromatography and single quadrupole mass spectrometry

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ABSTRACT

Background: We optimized a quantitative amino acid method with pre-column derivatization, norvaline (nva) internal standard and reverse phase ultra-performance liquid chromatography by replacing the ultraviolet detector with a single quadrupole mass spectrometer (MS_{nva}).
Method: We used $^{13}C^{15}N$ isotopically labeled amino acid internal standards and a C18 column with 1.6 μm particles to optimize the chromatography and to confirm separation of isobaric compounds (MS_{lis}). We compared the analytical performance of MS_{nva} with MS_{lis} and the original method (UV_{nva}) with clinical samples.
Results: The chromatography time per sample of MS_{nva} was 8 min, detection capabilities were < 1 μmol/L for most components, intermediate imprecisions at low concentrations were < 10% and there was negligible carryover. MS_{nva} was linear up to a total amino acid concentration in a sample of approximately 9500 μmol/L. The agreements between most individual amino acids were satisfactory compared to UV_{nva} with the latter prone to outliers and suboptimal quantitation of urinary arginine, aspartate, glutamate and methionine. MS_{nva} reliably detected argininosuccinate, β-alanine, citrulline and cysteine-s-sulfate.
Conclusion: MS_{nva} resulted in a more than fivefold increase in operational efficiency with accurate detection of amino acids and metabolic intermediates in clinical samples.

1. Introduction

Quantitative amino acid analysis in plasma, urine and cerebrospinal fluid is central to the diagnosis and therapeutic management of inherited disorders of amino acid transport and metabolism. The reproducible ninhydrin post column derivatization method with spectrophotometric quantitation described by Stein and Moore [1,2] has been the foundation of this analysis for nearly seven decades. Separation of the amino acids and related components was typically achieved with ion exchange liquid chromatography.

The relatively recent introduction of reverse phase ultra-performance liquid chromatography (UPLC) and 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbonate (AQC) [3] pre-column derivatization with norvaline (nva) as internal standard and ultraviolet (UV) detection has reduced the chromatography time from approximately 3 h to 45 min per sample [4]. Analysis of multiple amino acids in a single chromatography run remains a challenge due to their diverse chemical characteristics and the wide concentration ranges encountered in health and disease. Unpredictable interference can occur in some patients due to co-eluting exogenous compounds that also absorb in the UV spectrum e.g. gabapentin and histidine; aminoglycosides and phenylalanine; aminocaproic acid [5]. The incomplete resolution of rarely occurring endogenous metabolites such as argininosuccinate with ethanolamine and 1-methyl histidine with cysteine-s-sulfate can also be problematical and repeat analyses with altered chromatographic conditions are required to resolve them [6]. The throughput of a UV based method is therefore limited by the long chromatography times and the occasional need to repeat the analysis.

Abbreviations: UPLC, ultra-performance liquid chromatography; AQC, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbonate; nva, norvaline; UV, ultraviolet; MS, mass spectrometry; lis, labeled internal standards ($^{13}C^{15}N$ isotopes); Sd, standard deviation; csf, cerebrospinal fluid; PEA, phosphoethanolamine

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Tandem mass spectrometry (MS/MS) with its inherent analytical specificity has the potential to avoid the deficiencies of UV based methods. MS/MS methods with or without a derivatization step and using either a single unlabeled or multiple isotopically labeled internal standards (lis) have been described [7–9]. Isobaric compounds (leucine; isoleucine; alloisoleucine; proline, β-alanine; alanine; sarcosine, 1 and 2-methylhistidines and α-, β- and γ-butyrates) must be resolved either chromatographically or by a unique ion fragmentation pattern.

Single quadrupole mass spectrometry (MS) detectors are more affordable, robust, and relatively simple to operate and is a promising detection technique with analytical properties intermediate between UV and MS/MS [10]. The accurate mass detection may provide superior resolution to UV for co-eluting compounds with a different molecular mass, but the absence of ion fragments in this type of detector makes chromatographic separation of isobaric components paramount. A potential obstacle to MS detection is the lack of readily available isotopically labeled internal standards for each amino acid and related metabolite of interest in clinical specimens.

Our aim was to optimize an established quantitative amino acid method by using MS detection, streamlining sample preparation and optimizing the chromatographic conditions. We retained the AQC derivatization procedure and also confirmed the suitability of nva as a single isotopically unlabeled internal standard.

2. Materials and methods

As a proof of concept we swapped the UV detector of a commercial amino acid method (UV_{nva}) with a MS detector while keeping the sample preparation and chromatographic conditions unchanged. In a stepwise manner we optimized the sample preparation and the chromatographic conditions while using isotopically labeled amino acids as internal standards (MS_{lis}). To assess the suitability of nva as internal standard we compared this combination (MS_{nva}) to MS_{lis} as well as the original method with UV detection (UV_{nva}).

2.1. Instrumentation and reagents

The following items were sourced from Waters (Waters Corporation, Milford, USA): MassTrak AAA Solution kit, an Acquity UPLC system with a binary solvent manager, sample organizer and Empower data manager to perform UV_{nva} [4]. An Acquity QDa single quadrupole mass detector, a Cortecs UPLC BEH C18 column (1.6 μm particles; 2.1 × 150 mm), the neutral, acidic and basic amino acid standard solutions as well as allo-isoleucine, glutamine, nva and AQC reagents of the MassTrak AAA Solution kit were used for MS_{lis} and MS_{nva}.

We expanded the range of amino acids in the commercial calibrators by adding argininosuccinate, cysteine-s-sulfate and homocitrulline (all obtained from Sigma Aldrich, St Louis, USA) to the mixture. Working calibrators were prepared at concentrations of 100; 200 and 400 μmol/L for all the components with the exception of cystine and cystathionine which were present at 50; 100 and 200 μmol/L respectively. ^{13}C and ^{15}N isotopically labeled amino acids (Metabolomics Amino Acid Mix Standard) were obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA). The formic acid (Sigma Aldrich) and acetonitrile (Thermo Fisher, Waltham, USA) were MS-grade.

Two levels of ClinChek amino acid quality control material (Recipe, Munich, Germany) were used to investigate imprecision. External quality proficiency material was obtained from the European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism (ERNDIM, Netherlands) and the Australasian Society for Inborn Errors of Metabolism and the Human Genetics Society of Australasia (Sydney, Australia) education program.

2.2. Sample preperation

With UV_{nva} the samples, calibrators and controls were prepared according to the manufacturer's instructions [4]. To improve workflow we investigated whether we could perform the penultimate heating step of the original UV_{nva} after the final addition of 900 μL water. In the optimized procedure we added 25 μL of either the calibrator, quality control material or sample and 25 μL water to 50 μL of 200 μmol/L norvaline in 10% 5-sulfosalicylic acid. Ten [10] μL of supernatant obtained after centrifugation for 5 min at 10000*g* was added into 70 μL borate buffer in 0.2 mol/L NaOH. This was followed by adding 20 μL of the derivatization reagent and vortexing for 10 s. The volume was then made up to 1000 μL with water, vortexed for 10 s after which the vials were placed in a 55 °C heating block for 10 min.

2.3. Optimized chromatography and mass spectrometry conditions

The column temperature was 55 °C, flow rate 0.5 mL/min with the initial conditions of 99% mobile phase A (0.1% formic acid in water) and 1% mobile phase B (0.1% formic acid in acetonitrile). After 0.5 μL of sample was injected a linear gradient (curve 6) was applied to 87% A at 2 min, followed by 85% A at 4 min and 5% A at 5 min before returning to initial conditions from 7.6 to 8 min for re-equilibration. The ion source was a heated electrospray interface operated in the positive mode with the detector programmed to sample single ion reactions of selected atomic masses during timed intervals as depicted in Fig. 1. The probe temperature was 600 °C, the cone voltage 0.8 kV and the sampling rate 5 points/second.

2.4. Method validation

We characterized the detection capability, imprecision, carryover and linearity of the MS_{nva} method. The detection capability was estimated by analyzing samples with concentrations of 0, 1, 5 and 10 μmol/L ten times each in one run, plotting the standard deviation (Sd) against concentration and then estimating by extrapolation the Sd_{blank} and concentration where the imprecision was 20% ($LOQ_{20\%}$). Intermediate imprecision was determined over 20 days at two control levels. Carryover between samples was assessed by analyzing a blank immediately after a sample with high concentrations. Grossly elevated total amino acid concentrations in clinical samples may potentially exhaust the derivatizing reagent and result in non-linearity. We investigated this by analyzing 3 separate post mortem plasma samples, with extremely high total amino acid concentrations at several dilutions and then sequentially discarding the highest value before repeating the statistical analysis. We tested the linearity of the high calibrator and also for selected amino acids that were anticipated to occur at higher levels in clinical samples (glycine, histidine, threonine and serine).

We compared MS_{nva} to the MS_{lis} with 139 plasma, 100 urine and 97 cerebrospinal fluid (csf) samples submitted for routine amino acid analysis. After the optimization process was complete we compared MS_{nva} with UV_{nva} in another set of 80 plasma and 114 urine samples in order to standardize the time between sample preparation and analysis so that any artefactual conversion of glutamine to glutamate and arginine to ornithine was minimized. In our routine service delivery we use the UV_{nva} as originally described [4] and if interference is suspected in a sample we follow this up with a second analysis under alternative chromatographic conditions [6]. In the scatterplots and comparisons we only present results obtained with the original UV_{nva} and not the final value obtained with the further analysis. We tested the ability of MS_{nva} to deal with problematical samples by analyzing QAP and stored patient samples. The statistical analysis and non-parametric Passing Bablok regressions were performed with Analyse-it version 2.30 (Leeds, UK). Pearson's parametric correlation was used to assess the relative strengths of the associations.

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