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High-throughput quantitation of amino acids in rat and mouse biological matrices using stable isotope labeling and UPLC–MS/MS analysis☆

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ABSTRACT

Quantifying amino acids in biological matrices is typically performed using liquid chromatography (LC) coupled with fluorescent detection (FLD), requiring both derivatization and complete baseline separation of all amino acids. Due to its high specificity and sensitivity, the use of UPLC–MS/MS eliminates the derivatization step and allows for overlapping amino acid retention times thereby shortening the analysis time. Furthermore, combining UPLC–MS/MS with stable isotope labeling (e.g., isobaric tag for relative and absolute quantitation, i.e., iTRAQ) of amino acids enables quantitation while maintaining sensitivity, selectivity and speed of analysis. In this study, we report combining UPLC–MS/MS analysis with iTRAQ labeling of amino acids resulting in the elution and quantitation of 44 amino acids within 5 min demonstrating the speed and convenience of this assay over established approaches. This chromatographic analysis time represented a 5-fold improvement over the conventional HPLC–MS/MS method developed in our laboratory. In addition, the UPLC–MS/MS method demonstrated improvements in both specificity and sensitivity without loss of precision. In comparing UPLC–MS/MS and HPLC–MS/MS results of 32 detected amino acids, only 2 amino acids exhibited imprecision (RSD) >15% using UPLC–MS/MS, while 9 amino acids exhibited RSD >15% using HPLC–MS/MS. Evaluating intra- and inter-assay precision over 3 days, the quantitation range for 32 detected amino acids in rat plasma was 0.90–497 μM, with overall mean intra-day precision of less than 15% and mean inter-day precision of 12%. This UPLC–MS/MS assay was successfully implemented for the quantitative analysis of amino acids in rat and mouse plasma, along with mouse urine and tissue samples, resulting in the following concentration ranges: 0.98–431 μM in mouse plasma for 32 detected amino acids; 0.62–443 μM in rat plasma for 32 detected amino acids; 0.44–8590 μM in mouse liver for 33 detected amino acids; 0.61–1241 μM in mouse kidney for 37 detected amino acids; and 1.39–1681 μM in rat urine for 34 detected amino acids. The utility of the assay was further demonstrated by measuring and comparing plasma amino acid levels between pre-diabetic Zucker diabetic fatty rats (ZDF/Gmi fa/fa) and their lean littermates (ZDF/Gmi fa/?). Significant differences ($P < 0.001$) in 9 amino acid concentrations were observed, with the majority ranging from a 2- to 5-fold increase in pre-diabetic ZDF rats on comparison with ZDF lean rats, consistent with previous literature reports.

1. Introduction

Amino acids are the key building blocks of proteins and other essential biomolecules and hence represent a major group of endogenous compounds of interest in metabolite profiling in both preclinical and clinical settings [1,2]. Detection and accurate quantitation of amino acids play an important role in disease diagnostics, including diabetes [3,4] and diabetic ketoacidosis [5,6], kidney disease [7,8], inflammatory disorders [9] and cancer [10,11]. The traditional approach for amino acid analysis employs ion-exchange chromatography followed by post-column derivatization using ninhydrin and UV detection [12]. Other methods reported include use of pre-column fluorescent derivatization to enhance detection sensitivity followed by reversed-phase HPLC [13,14]. While these methods have been well established for routine amino acid analysis, they are limited in their abilities to offer

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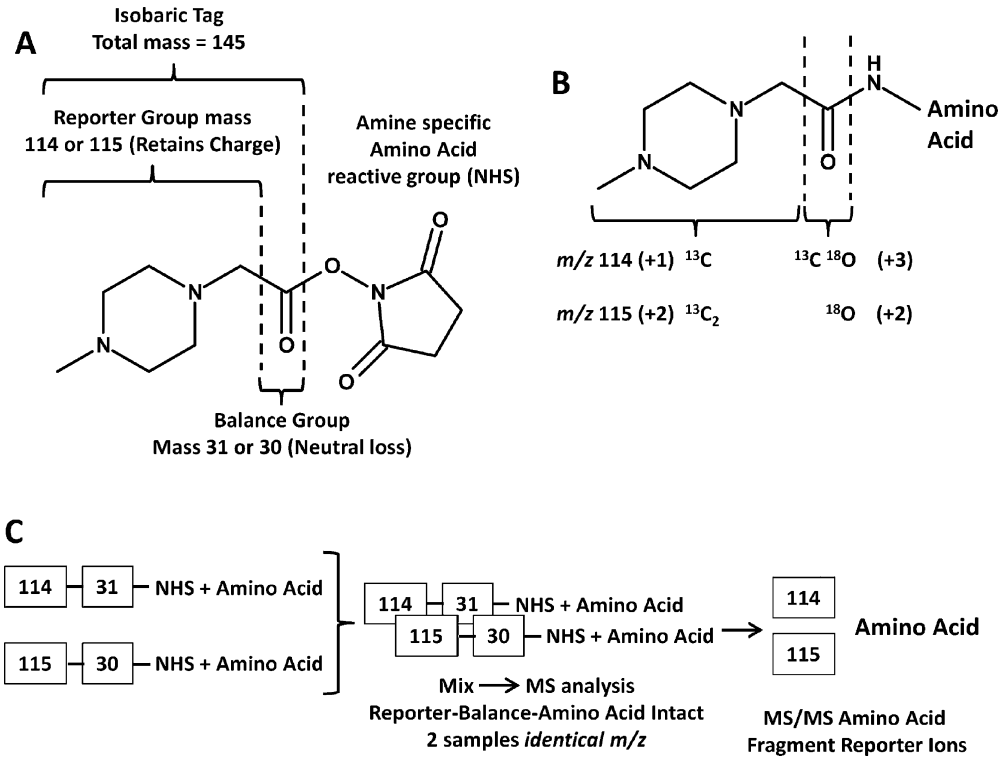

Scheme 1. (A) Description of the isobaric amino acid labeling reagent and tagging chemistry, consisting of a reporter group (based on *N*-methylpiperazine), a mass balance group (carbonyl), and an amine-reactive group (*N*-hydroxysuccinimide (NHS) ester). The overall mass of reporter and balance groups are kept constant using differential isotopic enrichment with ^{13}C and ^{18}O atoms. (B) Labeling reagents are linked to the amino group of amino acids. The reporter groups include *m/z* 114 (label for amino acid reference standards provided in the kit) and *m/z* 115 (label reacted with free amino acids in the samples), while the balance group ranges in mass from 31 and 30 Da, respectively, keeping the combined total mass consistent (145 Da) for each of the two labeling reagents. (C) Amino acid reference standards labeled with *m/z* 114 and samples labeled with *m/z* 115 are mixed prior to MS/MS analysis, resulting in detection of isobaric tag reporter ions at *m/z* 114 and 115 with resulting peak area ratios used for quantitative analysis. Information and diagrams have been previously published [19].

sufficient selectivity and sensitivity for samples in complex biological matrices. To address many of the challenges experienced when using the previously mentioned types of derivatization agents, while at the same time simplifying sample preparation and increasing throughput, recent publications have investigated the application of stable-isotope labeling coupled with LC–MS/MS [15,16]. One approach utilized iTRAQ (i.e., isobaric tag for relative and absolute quantitation) isotope labeling of physiological amino acids followed by LC–MS/MS quantitation [17]. This study was the first to compare iTRAQ stable-isotope labeling coupled with LC–MS/MS with the established approaches using GC–MS [18] and cation-exchange chromatography followed by post-column derivatization and UV detection [12]. Results from this study were shown to be similar, or superior to those from traditional methods.

The iTRAQ reagents are a set of isobaric reagents which are amine-specific, allowing for the identification and quantitation of multiple amine-containing components in the sample simultaneously, leading to their wide use in proteomics studies [19]. A schematic depicting the iTRAQ labeling reagents and their reactions with amino acids for detection is shown in Scheme 1. Two amine-specific isobaric reagents which possess two different reporter mass "tags" generated as MS/MS fragment ions are used for amino acid analysis. One reagent labels the free amino acids in the study sample extract (mass tag: 115). The second reagent consists of 44 different amino acids (mass tag: 114) of known concentrations, which are employed as internal reference standards. In LC–MS/MS analysis, both 115-labeled free amino acids and the 114-labeled known concentration internal standards appear at the same mass-to-charge ratio (*m/z*) and at the same retention time but possess different product ions which are quantitated in the multiple reaction monitoring (MRM) mode.

The application of ultra-performance liquid chromatography (UPLC) separation has demonstrated significant improvements compared with conventional HPLC, especially when coupled with mass spectrometry analysis. Utilizing sub-2 μm column particles and mobile phases at high linear velocities, and instrumentation that operates at higher pressures than those used in HPLC, drastic increases in resolution, sensitivity and speed of analysis can be achieved with UPLC. For example, reported applications of UPLC–MS/MS in metabolic profiling have demonstrated 10-fold increase in speed, a doubling of peak capacity, and a 3- to 5-fold increase in sensitivity compared with conventional reversed-phase HPLC [20–22].

Coupled with UPLC–photodiode array (PDA) and MS/MS analysis of amino acids, a more recent study reported the use of an alternative type of derivatization agent, i.e., AccQ·Tag (Waters Corporation, Milford, MA, USA), which was able to identify and quantify 16 amino acids in *Plasmodium falciparum* and 25 amino acids in human red blood cells [23]. The use of UPLC in this study provided a rapid LC separation time of less than 10 min, but required the use of both internally and externally generated calibration curves along with deuterated amino acids which served as internal standards. The goal of the present study was to develop a more rapid and selective UPLC–MS/MS method, when compared to the use of HPLC–MS/MS, coupled with iTRAQ-labeled amino acid analysis for the quantitation of amino acids in complex biological matrices. A modified