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Improving detection sensitivity of amino acids in thyroid tissues by using phthalic acid as a mobile phase additive in hydrophilic interaction chromatography-electrospray ionization-tandem mass spectrometry

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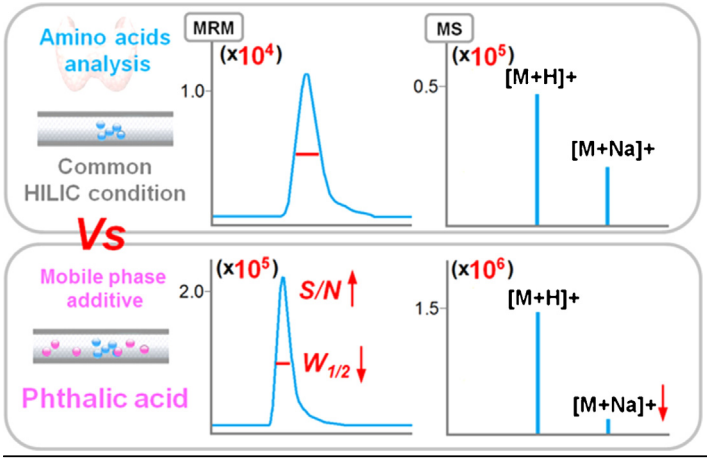
HIGHLIGHTS

- HILIC–ESI-MS/MS method was used to quantify 24 free AAs in human thyroid tissues.
- Addition of 0.08 mM of phthalic acid to the eluent enhanced the sensitivity of AAs.
- Narrowed peak shapes of AAs were achieved with phthalic acid in the mobile phase.
- The mechanism for the signal intensity enhancement by phthalic acid was investigated.

GRAPHICAL ABSTRACT



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ABSTRACT

In this work, 0.08 mmol L^{-1} of phthalic acid was introduced as a mobile phase additive to quantify free amino acids (AAs) by hydrophilic interaction liquid chromatography (HILIC) coupled with electrospray ionization tandem mass spectrometry (ESI-MS/MS). The addition of phthalic acid significantly increased the signal intensity of protonated AA ions, resulting from the decrease of the relative abundance of AA sodium adducts. Meanwhile, the chromatographic peak shapes of AAs were optimized. As a consequence, there was a noticeable increase in the sensitivity of detection for AAs. The limits of detection (LOD) and

Abbreviations: AA, amino acid; UHPLC, ultra-high-performance liquid chromatography; MRM, multiple reaction monitoring; ACN, acetonitrile; Phe, phenylalanine; Leu, leucine; Lle, isoleucine; Trp, tryptophan; Met, methionine; Val, valine; Pro, proline; Gln, glutamine; Tyr, tyrosine; Cys, cysteine; Ala, alanine; Gly, glycine; Thr, threonine; Ser, serine; Arg, arginine; Glu, glutamic acid; Asn, asparagine; His, histidine; Hpro, hydroxyproline; GABA, γ-aminobutyric acid; β-Ala, β-alanine; Asp, aspartic acid; Lys, lysine; Abu, 2-aminobutyric acid; Cit, citrulline.

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quantification (LOQ) of the AAs ranged from 0.0500 to 20.0 ng mL^{-1} and from 0.100 to 50.0 ng mL^{-1}, respectively, which were 4–50 times lower compared to the values measured without the addition of phthalic acid. The enhanced detection and separation of AAs were obtained by merely adding phthalic acid to the mobile phase without changing other conditions. Eventually, this simple method was validated and successfully applied to the analysis of twenty-four kinds of free AAs in human thyroid carcinoma and para-carcinoma tissues, demonstrating a significant increase of most AAs in thyroid carcinoma tissues ($p < 0.05$).

1. Introduction

Amino acids (AAs) are important building blocks of proteins and precursors of many essential biomolecules, which actively regulate key metabolic pathways and physiological processes that are necessary for health, growth, development, reproduction, and homeostasis of organisms [1]. Quantitative analysis of AAs from physiologic samples is critical for diagnosing and screening patients of a wide variety of inherited AA disorders. However, compared to other biological samples, such as blood and urine, the concentration variation of AAs in tissue samples (e.g, thyroid tissue) has not been extensively investigated [2].

A number of techniques for AA analysis have been developed [3], including nuclear magnetic resonance [4–6], liquid chromatography coupled with optical detection [7,8], gas chromatography-mass spectrometry [9,10], liquid chromatography–mass spectrometry (LC–MS) [11], and capillary electrophoresis [12,13]. Among the varieties of techniques, MS plays an important role in the analysis of protein and metabolite from complex biological samples due to the high sensitivity and selectivity [14–16]. Since most AAs show high polarity with low molecular weights, pre-treatment of chemical derivatization is often employed to achieve the increased retention on the reversed-phase column and desired detection sensitivity in LC–MS analysis [17–22]. However, this procedure is time-consuming and often leads to some problems, such as reagent interferences, side reactions, poor reproducibility and derivative instability. Thus, quantitation of AAs without derivatization by LC–MS method remains the superior advantageous of simplicity and flexibility [23].

One strategy that has been established for AA analysis is the addition of ion-pairing reagents (trifluoroacetic acid, tridecafluoroheptanoic acid, pentadecafluorooctanoic acid and so on) to the mobile phase to achieve efficient retention and separation of AAs on the reversed-phase column [24]. However, the reagents often suppress the signal intensity of analytes because of ion-pair formation, high conductivity as well as high surface tension of the eluent [25,26]. Another practical strategy to analyze AAs is HILIC method, which is suitable for MS detection and able to retain polar analytes without ion-pairing reagents. Recent studies related to AA analysis using HILIC have adopted formic acid/ammonium formate or acetic acid/ammonium acetate as buffer salts to acquire optimized peak shapes [27–29]. It should be noted that the detection sensitivity of AAs were often lower than those of the derivatized ones [30,31]. Therefore, the development of a simple but effective way to achieve comprehensively improved performance for AAs analysis is in demand.

Most analytes are found to generate both protonated ion peaks and analyte–metal adduct ion peaks by using ESI-MS in the positive ion mode [32]. It is a practical method to improve the detection sensitivity by reducing the AA–metal adducts, which leads to the enhancing of the protonated AA ions. As an extremely feasible method, utilization of mobile phase additives was supposed to have the same effect on signal intensity of AAs [33,34]. Thus, our goal in this work was to find an excellent mobile phase additive to enhance the analytical capabilities of HILIC–MS in the determination of AAs.

In this manuscript, we studied a series of acid additives to find useful mobile phase additives applicable to ESI-MS. Among them, phthalic acid stood out for the significantly enhanced AAs detection sensitivity and optimized peak shape in HILIC–ESI-MS/MS analysis, which was probably due to its ability to attach Na^+. Finally, this method was successfully applied to quantify twenty-four kinds of free AAs in biological tissues.

2. Material and methods

2.1. Chemicals

Acetonitrile (ACN) and methanol (MeOH) were of HPLC grade and purchased from Merck KGaA (Darmstadt, Germany). Formic acid (~98%, for mass spectrometry), ammonium formate (≥99.0%, HPLC), and phthalic acid (≥99.5%) were purchased from Fluka (Sigma–Aldrich, Milan, Italy). Ultrapure deionized water was purified with a Direct-Q water purification system (Millipore, El Paso, TX, USA). Standard AAs, phenylalanine (Phe), leucine (Leu), isoleucine (Lle), tryptophan (Trp), methionine (Met), valine (Val), proline (Pro), glutamine (Gln), tyrosine (Tyr), cysteine (Cys), alanine (Ala), glycine (Gly), threonine (Thr), serine (Ser), arginine (Arg), glutamic acid (Glu), asparagine (Asn), histidine (His) were purchased from Shanghai Aladdin reagent Co., Ltd. Hydroxyproline (Hpro), γ-aminobutyric acid (GABA), β-alanine (β-Ala), aspartic acid (Asp), lysine (Lys) were purchased from Aldrich (Milwaukee, WI, USA). 2-aminobutyric acid (Abu) and citrulline (Cit) were purchased from Tokyo Chemical Industry Co., Ltd. The purity of each compound was more than 98%, determined by HPLC analysis. L-phenyl-d_5-alanine (Phe-IS) and L-alanine-3,3,3-d_3 (Ala-IS) were purchased from Sigma–Aldrich. L-serine-2,3,3-d_3 (Ser-IS) and L-glutamic acid-2,3,3,4,4-d_5 (Glu-IS) were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). Other reagents and chemicals were of analytical grade.

A mixed standard stock solution containing all the standard compounds was prepared by dissolving them in water/acetonitrile (1:1, v/v), and the concentrations of these analytes were 100 mg mL^{-1}. The exception was Tyr at 50 mg mL^{-1}. Then the mixed standard stock solution was diluted with water/acetonitrile (1:1, v/v) to provide working solutions with different concentrations. Internal standard (IS) solution containing four stable-isotope labeled AAs was also obtained by dissolving them in water/acetonitrile (1:1, v/v), and the concentrations of these analytes were as follows: Phe-IS and Ala-IS, 10 μg mL^{-1}; Ser-IS and Glu-IS, 100 μg mL^{-1}. It was used to prepare the required solutions just prior to analysis. Both AA standard solutions and IS solutions are stored at -20 °C.

2.2. LC–ESI-MS/MS analysis

UHPLC analysis was performed on a Nexera UHPLC system (Shimadzu, Tokyo, Japan), equipped with a system controller (CBM-20A), a binary solvent delivery system (LC-30AD), an autosampler (SIL-30AC), and a column heater (CTO-30A). HILIC separation was performed on an ACQUITY UPLC BEH Amide