REVIEW ARTICLE

ANALYSIS OF AMINO ACIDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT
Amino acids are the building blocks of proteins and are considered as the key precursors for the formation of hormones and low molecular weight nitrogenous substances with biological importance. Since the analysis of amino acids has been carried out for both qualitative and quantitative purposes with an aim to study their levels in the plasma concentration, the quantitative determination, in particular, also helps in the diagnosis of different diseases associated with their deficiency. This review article deals with the determination of amino acids by chromatographic methods which include ion-exchange chromatography (IEC), high performance liquid chromatography (HPLC), reverse phase-high performance liquid chromatography (RP-HPLC) and ultra-performance liquid chromatography (UPLC). The review will also give an idea for the preparation of samples, derivatization methods for the analysis of amino acids (direct and indirect methods) and separation of amino acids by high performance liquid chromatographic technique.

Keywords: HPLC, amino acids, derivatization, plasma.

1. INTRODUCTION
The organic compounds required by the body for the synthesis of proteins are termed as amino acids (AA), which are necessary to perform all the biological activities within the body1. There are more than five hundred AA that have been discovered by the scientists up till now. However, translation, a process of protein synthesis, runs efficiently with only twenty-two amino acids2. The human body is restricted to produce only few AA while the rest are obtained from food products that are rich in proteins. Apart from protein synthesis, these macromolecules are also involved in cellular metabolism, supply of energy and may serve as chemical messengers in transferring information between the cells3,4.

AA are zwitterions. They possess at least one amino group and bears a positive charge upon protonation while one carboxyl group which forms an anion when loose a proton5. The AA differs from each other with respect to their side chain as well as the interaction with the stationary phase is also dependent on the nature of the of side chain6.

2. ANALYSIS OF AA IN PLASMA
2.1. Purpose
The fundamental aim of AA analysis in plasma is to obtain a better understanding for the diagnosis of a disease. There are various diseases associated with either high level or low level of AA7,8. The increased level reveals the inherited metabolic disorders, usually seen in infants. Some of the main defects may include AA metabolism, organic acidurias and mitochondrial defects. However, low level of AA indicate nutritional deficiencies, fever and certain medical conditions9,10.

2.2. Preparation of Plasma
The AA are generally released from the plasma prior to its analysis. For this purpose, various methods

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have been employed so that each AA could be analyzed with high specificity and sensitivity. The preparation of plasma AA sample is directly linked up with the type of chromatographic technique utilized. For better results, it is highly recommended to minimize the sample preparation steps\(^{11}\). In the first step, the deproteination or protein precipitation is carried out to release the free AA\(^{12-15}\) by using different solvents such as perchloric acid, sulfosalicylic acid (SSA) and ethanol\(^{13,14}\). However, certain scientists have proved the benefits of perchloric acid over SSA because it does not cause any interference and also have no profound effects on the quantitative results of AA\(^{15,16}\). After the release of AA, the plasma samples are extracted, vortex and centrifuged. The collection of supernatant is followed by the filtration of the sample, maintaining the pH of the sample by adding appropriate buffers and placing them into vials for analysis\(^{15,17}\).

3. DERIVATIZATION

The basic purpose of derivatization step is to render the analyte to be detected with higher selectivity and sensitivity. The polar substances are converted to more stable forms under heat in order to have enhanced chromatographic properties and eventually improve their overall chromatographic behavior\(^{18,19}\). Since the AA are difficult to be detected in the chromatographic system due to the lacking of a strong chromophore or fluorophore in their structures, therefore, their indirect detection may be carried out by ultraviolet-visible (UV) or fluorescence detectors\(^{20-22}\).

The AA are derivatized by either direct or indirect methods. The direct method determines the AA without involving the derivatization procedure while indirect method is carried out by converting them into stable derivatives that are easily detectable by spectrometric detectors.

3.1. Indirect Method

The indirect analysis of AA is further categorized into pre-column and post-column derivatization.

3.1.1. Pre-column derivatization

This method involves the derivatization of AA prior to the injection into the chromatographic system and a reverse phase HPLC system with pre-column derivatization is applied. A better resolution as well as high sensitivity is efficiently achieved by this method along with shorter time of analysis i.e. 9.5 min\(^ {23}\). This method consumes less derivatization reagent thus allows the use of expensive ones. However, it involves increased labor cost as well as reduced reaction efficiency due to sample matrix\(^ {24}\).

3.1.1.1. Pre-column derivatization reagents

The AA analysis is effectively carried out by using pre-column derivatization reagent i.e. o-phthalaldehyde (OPA) which is the most widely used reagent in pre and post column derivatization. It is a low cost reagent with relatively low background signal, high selectivity and sensitivity. One of the major drawbacks in its use is that it forms unstable adducts and require mercapto-compound to be used as a co-reagent which eventually makes the procedure quite expensive. However, some authors have evolved the use of 9-fluorenlymethyl chloroformate (FMOC-Cl) as another derivatizing reagent\(^ {25}\). The reaction is carried out under mild conditions in aqueous solution and the resultant AA derivatives thus produced are highly stable and fluorescent. The limit of detection (LOD) by using this reagent in RP-HPLC is in low femto mole (fmol) range\(^ {26,27}\). The benefit of using FMOC-Cl for the purpose of derivatization is that the reaction becomes fast and simple but it also utilizes a hydrophobic amine such as 1-amino-adamantane hydrochloride to remove the excess reagent, thus increasing the cost of the reaction\(^ {26}\). Another reagent which is being used is phenylisothiocyanate (PITC). The PITC assures the analysis of submicrogram of AA samples rendering an accurate and reproducible quantitation from the complex biological matrices. Moreover it also allows shorter time of analysis\(^ {27}\) but the derivatization procedure is time consuming as excess of reagent has to be removed before the analysis\(^ {28}\).

3.1.2. Post-column derivatization

The ion exchange HPLC with post-column derivatization (PCD) is the earliest method applied for the analysis of AA. In this method, the separation
of the desired analyte is carried out. This is followed by the derivatization step which is an automated online procedure in which mixing of the suitable post-column reagent takes place leading to the detection of the compound of interest\textsuperscript{29}. As the derivatization procedure is automated in PCD, it enhances greater reproducibility and good quantitation. However, this method offers reduced sensitivity and the broad range of derivatizing reagents is also restricted. Moreover, this method requires longer run times achieving low throughput\textsuperscript{30}.

3.1.2.1. Post-column derivatization reagents
The most popular reagent used by the scientists in the ion exchange chromatography separation is ninhydrin, because of its easy availability and simple derivatization steps. It results in the formation of colored derivatives which are detected by the UV-vis spectrophotometer at 405 and 570 nm for secondary and primary amino compounds respectively\textsuperscript{31}. However, it undergoes a series of complex intermediate reactions that prolongs the derivatization procedure. Moreover, the resultant derivatives and ninhydrin solution itself are unstable and require additional reagent to make it stable thus, increases the cost of the analysis\textsuperscript{29,32}.

3.2. Direct Method
This method does not involve any derivatization step for the determination of the AA present in a sample rather they are measured directly. In order to prevent the delay in the diagnosis of metabolic diseases and for more accurate and reliable results, scientists have evolved the use of more specific and sensitive technique such as HPLC-ESI-MS/MS. The detection through MS/MS is also used to determine the trace contents of AA efficiently present in various complex physiological fluids such as plasma and urine\textsuperscript{21,33}.

3.2.1. Separation by HPLC
The separation is successfully carried out on an octadyl HPLC column using a volatile mobile phase and tridecafluorohexanoic acid (TDFHA) as an ion pairing reagent because it allows good AA separation and detection with higher sensitivity\textsuperscript{34}.

3.2.2. Detection by ESI-MS/MS
The peaks of underivatized AA are recorded on a positive mode ESI-MS/MS detection system. The lower limit of quantitation (LOQ) was found to be under the range of 250 fmol to 50 pmol which is far greater than LOQ of fluorescence derivatization i.e. 10 pmol\textsuperscript{35-38}.

4. DETERMINATION OF AA
4.1. Ion Exchange Chromatography
The ion exchange chromatography (IEC) has been considered as a classical technique for the determination of AA in physiological fluids with post column ninhydrin derivatization. This technique is widely applied for the analysis because of excellent separation and reproducible results accompanied by simple sample preparation\textsuperscript{39}. The IEC is popular despite being not suitable for high sample throughput and is rather time consuming. Moreover, the method lacks the ability to separate certain substances and the accuracy of quantitation is affected by the occurrence of numerous artifacts\textsuperscript{40}. Such circumstances can be reduced by using HPLC.

4.2. Reverse Phase (RP)-HPLC and Fluorescence Detector
A simple RP-HPLC method with high throughput has effectively been applied for the determination of most plasma AA. This method utilizes simple sample preparation and least sample volume that makes it more unique and widely used. In addition, it provides reproducible outcomes and good precision. The separation occurs by gradient elution RP-HPLC\textsuperscript{41} and involves the rapid automated online pre-column derivatization with OPA which reacts with AA to form highly fluorescent products. The products are easily detectable by fluorescence detector which employs an excitation wavelength of 340 nm and an emission wavelength of 455 nm. The limit of detection for each AA was found to be 38 fmol\textsuperscript{42}.

4.2.1. Drawbacks of RP-HPLC
Perfectly clean samples are pre-requisite for the separation by HPLC grade columns otherwise they would be destroyed or the presence of impurities
may result in inappropriate derivatization.\textsuperscript{43}

4.3. Chiral HPLC
Pasteur laid the foundation of optical isomerism and highlighted the important relationship between the chirality and the biological activity of the compound. All of the 20 common alpha amino acids except glycine possess a chiral carbon adjacent to carboxyl functional group which leads to the basis of a pair of enantiomers.\textsuperscript{44} The determination of L-AA is predominantly carried out by using HPLC technique followed by the detection through UV-vis or fluorescence detector, however, the occurrence of D-amino acids is limited in nature.\textsuperscript{45}

4.4. Ultra Performance Liquid Chromatography
The HPLC has been transformed into ultra-performance liquid chromatography (UPLC). The quantitative analysis of underivatized AA are successfully done by applying UPLC-MS/MS technique. The method has proved to be more competent providing high throughput, low sample volume consumption and maximum capacity than HPLC. In addition, it is highly reliable, robust and simplified in nature. However, overall it is considered as an expensive technique.\textsuperscript{46}

5. SEPARATION OF AA
The separation of AA are taken into account by two main approaches i.e. indirect chiral method where the interaction between AA and chiral derivatizing reagent (CDA) results in the formation of diastereomers. Those diastereomeric derivatives are separated on an achiral stationary phase as illustrated by Iliš et al.\textsuperscript{43} while direct chiral method is governed by the use of chiral stationary phase.\textsuperscript{47,48}

5.1. Merits and Drawbacks of Indirect and Direct Method
Indirect method is relatively simple, cost effective, and allows good chromatographic properties of derivatives with enhanced selectivity and sensitivity as compared to the direct method. However, it is a time consuming process along with the chances of racemization and kinetic resolution. Furthermore, interference with excess reagent may result in broad peaks and is therefore, not applicable to preparative analysis. In contrast, direct method requires simple sample preparation and is suitable for preparative purposes.\textsuperscript{49}

6. DETECTION OF AA
6.1. Use of Ultraviolet-Visible or Fluorescence Detector
The chromatographic separation coupled with pre- or post-column derivatization enabling the detection by UV-visible or fluorescence detectors is the most appropriate method. The UV or fluorescence detectors offer the advantages over electrochemical, chemiluminescent nitrogen and mass spectrometry detectors because of low cost and easy availability in the routine analytical laboratories.\textsuperscript{20,22,42}

6.2. Use of Mass Spectrometry
The ideal amalgam of HPLC and MS resulted in a major breakthrough in separation and detection of AA, which is more efficient and accurate than ever before as compared to any other combination technique. This hyphenated method offers its contribution in neonatal screening and early diagnosis of various metabolic disorders projected by Carpenter and Wiley.\textsuperscript{46} Furthermore, there are many types of mass spectrometers which are coupled with HPLC and provide the advantages and disadvantages of their own. However, in general, the outcomes of this coupled technique are high throughput, high selectivity because of selected mass, information on molecular mass, structural configuration and accurate quantitative analysis. In contrast, it is difficult to analyze non-volatile compounds due to the requirement of pure analytes.\textsuperscript{50-53}

7. CONCLUSION
A variety of techniques have been discussed but no diagnostic technique is found to be perfect as each method has its own unique ability to analyze the AA along with the unhidden drawbacks. However, it is widely accepted in publications that by utilizing liquid chromatography hyphenated with spectrometric detectors, a good quantitative analysis of AA can be achieved.
REFERENCES


39. Waterval WA, Scheijen JL, Ortmans-Ploemen MM, Habets-van der Poel CD, Bierau J. Quantitative UPLC-MS/MS analysis of underivatized amino acids in body fluids is a reliable tool for the diagnosis and follow-up of