Simultaneous estimation of Amino acids by using HPLC

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REVIEW ARTICLE

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ABSTRACT

Various methods for the individual as well as simultaneous estimation of amino acids using various techniques like HPLC and other way outs like electrophoresis have been described in this review paper. The amino acid determination by using HPLC can either be done by using pre-column or post column derivatization. The amino acid is first derivatized into a particular derivative and then is analysed into the column in the case of pre-column derivatization, whereas in the case of post column derivatization, the amino acid is first passed through the column for the sake of separation and then the separated amino acids are derivatized into their such derivatives which can be detected by fluorescence detector. Out of the above two mentioned techniques, pre-column derivatization is used more oftenly than the post column derivatization. Few of the most commonly used derivatization agents are phenylisothiocyanate, o-phthalaldehyde+2-mercaptoethanol, dansyl chloride, phenylthiohydantoin etc.

Keywords: Post column derivatization, pre column derivatization, amino acids, electrophoresis.

1. Introduction

Amino acids are the biochemical compounds that result from the acid catalysed hydrolysis of proteins and polypeptides. A single amino acid and/or polypeptide can yield a variety of amino acids, but only 20 amino acids are such which occur from the naturally occurring polypeptides and they are referred to as standard amino acids. They are the structural building blocks of proteins. A huge portion of the natural amino acids is found in the form of their laevorotatory isomer (1).

Amino acids are responsible for the successful performance of various physiological and mental functions of the body. The deficiency of even one of the amino acids in the body can lead to sever problems associated with bodily functions as well as can cause mental or psychological disorders. Supporting this statement, TJ de Koning stated and proved that the deficiency of serine, which is a non-essential amino acid, can cause severe psychological disturbances and can create problems like seizure. The root cause of these problems or of serine deficiency is the deficiency or scarcity of certain enzymes that are necessary for the biosynthesis of serine in the body, which are, 3-Phosphoglycerate dehydrogenase and 3-Phosphoserine phosphatase. The deficiency becomes the main cause for the occurrence of certain symptoms like congenital microcephaly, sever psychomotor retardation, unrecognized seizures etc. which can be overcome by L-serine administration or by glycine therapy (2).

Similarly, the deficiency of other amino acids also causes some or the other problems, severity depending upon the extent of the deficiency. For example, the deficiency of glutathione, as put forward by Perry Thomson L and David V et al also causes severe psychomotor imbalances and disturbances. Glutathione (GHS) is one of the most important amino acids because it is the only natural antioxidant formed in the body. Its main location in the body form where it performs the entire antioxidant activities is the

substantia nigra of brain and any kind of hindrance in the production of glutathione in the brain results in the oxidative degradation of L-DOPA and dopamine which triggers the formation of very unwanted reactive oxygen free radicles and other reactive species that are responsible for causing an immense damage to the inner and outer brain membrane. Hence, less production or impaired formation of GSH in the brain will ultimately destroy the dopaminergic neurones that take the patient into a slowly spreading aggressive condition known as Parkinson´s disease (3).

HPLC- An Introduction

Since the day HPLC came into play, it has enabled the scientists and analysts with the most recent and advanced technology that helps in the quantitative as well qualitative analysis of a huge diversity of biochemical as well as chemical synthetic compounds. The separation of compounds using HPLC in the conventional times was performed either by liquid-liquid partition chromatography or by liquid-solid adsorption chromatography. The liquid-liquid chromatography was proved to be disadvantageous over the liquid-solid one because it is very troublesome and hectic to stick a liquid stationary phase on the column and to maintain it properly on the solid column throughout the analysis while another liquid which is a mobile phase is already flowing over it. There was another trouble which was being faced due to the liquid-liquid chromatography that every time we had to saturate the stationary phase with the mobile phase and this phenomenon is called as pre-saturation .because of this restriction, the practitioners were only able to use isocratic elution techniques back then and were not able to practice gradient elution.

The technique of High Performance Liquid Chromatography (HPLC) was introduced in the start of 1960s. This analytical technology has made it easier to analyse different components of a single complex mixture without the risk of being contaminated and without the chance of any error in the detection. The main principle involved in the technology is adsorption, and the elution properties of the components depend on the affinity of each component towards the stationary as well as the mobile phase. The components which are more partitioned towards the stationary phase will be stuck in that phase and hence will be eluted out lately as compared to the ones that are more attracted towards the mobile phase. In turn, adsorption chromatography is also of two types:

- Normal phase: In the case of normal phase chromatography, the solvent system involved is non-polar (such as Hexane) and the adsorbent is polar (such as Silica).
- Reverse phase: In this chromatography, more polar mobile phase is preferred (like Water, methanol) than the stationary phase which is non- polar (like Hydrocarbons).

HPLC is a far better approach than the primitive types of chromatographic techniques because in HPLC, the overall pressure for the flow of the mobile phase is enhanced with the help of pressure generating pumps and this pressure combined with force of gravity gives an add on to the pressure which increases the flow rate of the mobile phase through the column to many folds hence increasing its overall potential (4).

Nowadays, various latest stationary phases are in use for the purpose of performing the HPLC. Some of them are discussed below -:

- **Early bonded phases:** These types of stationary phases are such which consists of certain chemical groups in them that firmly bind to the silica backbone of the column.
- **Silicoxane stationary phases**: One of the highly used stationary phases, these are formed by the reaction of the silanol group of the silica skeleton of the column with a special kind of organosilane compounds.
- **Polymeric stationary phases**: This type of stationary phases involve polymerization reactions in their making. These reactions are carried out between the silica backbone and various monomers.

Various new stationary phases have been developed in the recent times due to the advancements that have taken place in the industry. Due to such advancements, the use of conventional C8 and/or C18 columns have seen a certain decline over a past few couple of years (5).

Amino acid estimation- An overview

YV Teckerkas and AD Denisenko were amongst the first few investigators who developed a method for the estimation of various amines and amino acids in the body. This was a serious breakthrough in the studies

related to amino acids because with the advent of such methods, it became easy to measure the concentration of various amino acids in the body and hence, their deficiency or excess in the plasma could be detected and diseases could now be identified at a very early stage. Because of such investigations, it was known that an unusual increase in the level of plasma homocysteine can result into sever health issues like cardiovascular diseases and cerebrovascular diseases etc. (6).

In the recent days, the estimation of amino acids can be easily carried out with the help of a chemical reaction called as Derivatization reaction. Earlier approaches used for the estimation were the ion exchange chromatography and then the performance of derivatization by using certain reagents like o-phthalaldehyde, 2-mercaptoethanol and so on. This technique of derivatization was followed by the reverse phase HPLC of the derivatized samples. This method showed an excellent result in the estimation and detection of amino acids with higher efficiency, easy usage and excellent flow rates. Early reagents for derivatization were less efficient as they were not able to demarcate the different amino acids in the complex mixture. So new reagents were introduced namely, o-phthalaldehyde + 2-mercaptoethanol which reacted with primary amines to form very effective derivatives (7).

The gradient elution technique was in use for the estimation work for a very long time but it showed a considerable drawback that it was showing a highly unstable baseline which created problems in the analysis work. This was the reason to introduce isocratic elution technique that was simple and accurate (8).

2. Techniques of derivatization

Pre column derivatization

Brian A. Bidlingmeyer et al described pre column derivatization as an efficient method for the rapid analysis of the amino acids. This technique involves the derivatization of amino acid before its entrance into the column. Once the derivatives are formed, they are pumped into the high performance columns (reverse phase). This technique is called as the pre column derivatization. The agents that are used often for the estimation are the dansyl derivatives and the phenylthiohydantoin derivatives. They were having the properties that were needed by them to be a good derivatization reagent but they also possessed some limitations like lack of derivative stability or interference with reagent peaks. O-phthalaldehyde and 2-mercaptoethanol was another discovered agent for the same purpose of derivatization. It solved various difficulties in the estimation task but the only drawback it offered was its non-reactivity towards the secondary amines and secondary amino acids. Furst P. and Pollack L. et al performed the separate analysis on the biological fluids for their amino acid estimation. They carried out the analysis using all the three different reagents like, dansyl chloride, o-phthalaldehyde and 2-mercaptoethanol and phenylisothiocyanate.

O-Phthalaldehyde method:

This was bought into the market in 1971 and is the most widely accepted reagent.

METHOD-: Fluorescence detectors were used for the detection and the derivatives were prepared manually. The problems encountered with the use of 2-mercaptoethanol was tackled by the use of an alternative derivatizing agent named 3-mercaptopropionic acid and acetonitrile. By the help of this reagent, the analysis work can be done in a time interval of 13 minutes. This helps in the estimation of amino acids in the liver, kidneys and muscles.

a. PITC method:

Method: This reagent is also called as Edman's reagent. The amino acids are derivatized by reacting them with the reagent and converting them into their thiocarbamyl derivatives. Once, the derivatives are formed, they are then sent to the column for further analysis. The thiocarbamyl derivatives are stable for several days under a refrigerated temperature.

b. Dansyl chloride method:

Method: The Dansyl chloride derivatives of all primary and secondary amino acids are very widely accepted for the research and estimation work. These derivatives can be detected by using fluorescence detectors. The time required for the derivatization to be completed is between 35 to 50 minutes if the reaction is carried out in dark. After that, the derivatives are ready for the analysis work in the RP-HPLC (9).

Rainer Schuster was the one who discovered a simpler 2 step pre column derivatzation technique for the analysis of all the primary and secondary amino acids altogether. All the previously described derivatizing agents were having one or the other problems in their usage which made them slightly incompetent and less efficient to be used in the estimation studies. On the other hand, this automated 2 step pre column derivatization was a separate simpler alternative and it had no such problems as were in the case of other derivatizing agents. In the first step of this technique, the primary amino acids are reacted with the o-phyhalaldehyde + 3-mercaptopropionic acid reagent. The next step involves the reaction of the secondary amino acids with the FMOC reagent (9-Fluorenylmethyl-chloroformate). Now, a mixture of both primary as well as secondary amino acids is ready which is introduced into the reverse phase column for the separation. It takes around 15-20 minutes for the estimation to be completed.

Method: This is a completely automated procedure of derivatization. The samples and the reagents are collected, transferred into a common vial and are mixed thoroughly.

Post column derivatization

The other technique which is a little primitive but once most widely one is the post column derivatization. It involves the separation of the analyte from the mixture and then that component or compound is moved into the column for the analysis. This post column approach is known as the offline post column derivatization. The online post column derivatization technique involves a separate reaction chamber for the reaction between the samples, reagents and the mobile phases. The reactions in this derivatization don't need to be flawless, which is huge advantage of this technique.

Disadvantages of post column derivatization-:

- The need of an external hardware system limits the use of this technique.
- Time and temperature of the reactions are not flexible.
- The resolution of the chromatogram will be lost after the mixing (11).

The post column derivatization, three main derivatization agents are widely preferred which are, o-phthalaldehyde, ninhydrin and fluorescamine. OPA and ninhydrin are two agents out of the three that are majorly used for the estimation reactions. The former two are used in the 90% of the cases while fluorescamine is used only in the remaining 10% of the cases because it does not react with the secondary amino acids. The use of fluorescamine as the derivatizing agent was developed by Unfriend et al. Fluorescamine reacts with the primary amino acids in the presence of a pH 9 buffer and the derivatives are detected by a fluorescence detector in the RP-HPLC. The disadvantage of the post column derivatization that it requires a separate reaction coil can be removed by decreasing the reaction time to the order of a few seconds. Recently, some new type of columns called as the AFT (Active Flow Technology) columns have been used in the post column derivatization. Because of these columns, the need of a separate reaction coil has been eliminated for the mixing of the analyte and the reagent. These columns allow the mixing of all the species like the reagents and the samples within themselves so, in a way; they are time saving and effective columns for the post column derivaatization (12).

3. Recent developments

As the facts mentioned above, technique of post column derivatization was not accepted much and researchers had to move towards the pre column derivatization. It was in 1994, when Hong Ji Liu introduced a novel derivatization reagent named as 6-amino quinolyl-N-hydroxysuccinimidyl carbamate (AQC) that possessed all the qualities of an ideal derivatization agent that were not present in the then available agents. AQC became the most preferred derivatizing agent because it had all the qualities of an ideal agent and it was able to react with all the primary as well as secondary amino acids successfully at an excitation wavelength of 250 nm and the emission wavelength at 395 nm with the help of fluorescence detectors and UV detectors (13).

In 2009, C Bueneo et al described another fact which led to a very easy and quick determination of amino acids. They proposed that several aromatic free amino acids like tryptophan, tyrosine and phenylalanine can be determined without the aid of derivatization as they are the compounds exhibiting natural

fluorescence. This was one of the most important facts proven because all the three mentioned amino acids are very important for the body in general. Tyrosine, being a non-essential amino acid is synthesized into the body and is useful in normal body temperature regulation and is a CNS stimulant, phenylalanine is an essential amino acid is useful in the stimulation of CNS and also plays an important role in reducing the symptoms of depression and other CNS related disorders, tryptophan is one of the most important amino acid as it is the precursor of vitamin niacin as well as of neurotransmitter serotonin. There are various established methods for the determination of these amino acids like ion exchange chromatography, gas chromatography and the most suited and preferred HPLC analysis.

Mark C Roach and Marlin D Haromny in 1987 developed a method for the estimation of amino acids by HPLC analysis by using a technique called as laser induced fluorescence by using a non-conventional reagent called as 2,3-Naphthalinedialdehyde. As we know that a UV region light was used for the determination in the case of o-phtalaldehyde, in the case of NDA, a visible region light of wavelength 457.9 nm was used. The conventional method of derivatization with the OPA reagent detected the amino acids up to a range of 100 fmols. To reduce the detection limit of the amino acids, a new derivatization reagent and a new technique called laser fluorescence technique was developed called as laser fluorescence technique. This involved a laser as a fluorescence source of the amino acids derivatives of NDA. From the starting days of this technique, it has shown a decrease in the detection limits of the amino acids up to 10 fmols (14).

Method to perform HPLC-LIF (HPLC-Laser Induced Fluorescence) Detection Method or procedure to derivatize with OPA (o-phtalaldehyde):

- The amino acid solution was taken from the stock solution and was then thoroughly mixed with the specified amount of the derivatization agent solution.
- After leaving the solution mixture undisturbed for about a minute, a sodium acetate buffer of pH 7 was added to it whose concentration was 1 molar and the total volume was made up to three ml.
- The sample was then allowed to get mixed properly and then was run in the column for the estimation to be completed.

Method or procedure to derivatize with NDA (2,3-Naphthalinedialdehyde):

- The standard amino acid solution was taken from the stock solution and a 100 fold excess quantity of cyanide was mixed to it. After that, an alkaline borate buffer of pH 9.5 was added to the solution mixture whose concentration was about 0.1 molar.
- Next step was to add another 100 fold excess of NDA reagent to the solution mixture and was allowed to mix properly.
- The solution was then allowed to stand undisturbed for about 15 minutes and then a required quantity of distilled water was added to it to make up the final volume.
- After all this preparation, the solution was run in to the column and the estimation was done using the usual HPLC derivatization technique (15).

OPA derivatization of honey and wine samples by HPLC pre column derivatization:

V Pereira et al in 2007 described a novel method for the simultaneous estimation of free amino acids in the wine and honey samples using in loop o-phthalaldehyde derivatization procedure. The derivatization of the samples was done with OPA in the same injection loop. Two mobile phases were used for this purpose; mobile phase A was- 10 mM sodium phosphate buffer of pH 7.3, methanol and tetrahydrofuran (91:8:1) and mobile phase B was; methanol and phosphate buffer (80:20). There are various analytical techniques for the simultaneous estimation of amino acids in these samples like ion-exchange chromatography, gas electrophoresis, HPLC etc. but the most successful and most widely used technique nowadays is the liquid chromatography along with mass spectroscopy that is also called as LC-MS/MS.

The only drawbacks of this technique are its cost and its restriction to be used only on the biological samples. That is the reason that this technique is not used so frequently. Again, the most accepted method for the determination of amino acids in the wine and honey samples is

the HPLC method after its derivatization with the proper derivatization agent. The usually preferred agents for this purpose are OPA, FMOC-cl, PITC etc. O-phthalaldehyde reagent is one of the most widely used and preferred agents because it serves nearly all the properties and advantages of an ideal derivatizaing reagents but has limitation that it cannot react with the secondary amino acids, but still it is one of the widely chosen reagents for the derivatization procedure as far as primary amino acids are concerned. It offers another benefit that nearly o other reagent offers is that when using this reagent, there is no need to purify the sample and also there is no need of a special separate extraction procedure to be followed which has to be done in the case of other reagents. The time taken by the OPA reagent to complete the process of derivatization is about 3 minutes. The time taken by FMOC reagent was about 6 minutes that is double the time taken by OPA as described by Bauza et al. But the method used by Krauze et al required about 20 minutes as they used dabsyl chloride as the derivatization agent (16).

Derivatization of D- and L- Aspartic acid followed by the Reverse phase HPLC (RP-HPLC)

Dana W Aswad in 1983 gave a simple method for the estimation of both the D- as well as L-isomer of aspartic acid by using o-phthalaldehyde and an optically active fluoregen called as N-acetyl-L-cysteine followed by the analysis of derivatives by using the reverse phase HPLC. Several methods are available for the simultaneous estimation and quantification of enantiomers of amino acids from their racemic mixture but the most commonly accepted method is that of Manning and Moore in their method, they derivatized a racemic amino acid mixture by allowing it to react with N-carboxy anhydride of an optically pure amino acid. The subsequent analysis of these derivatized amino acid racemic mixtures is done by usual reverse phase HPLC. Post their derivatization and analysis by HPLC, they are detected by fluorescence detectors. This method serves as an alternative to the use of an adduct called 2-meracaptoethanol which is an expensive adduct hence makes the process of derivatization and process of analysis more tough, troublesome and restricted. Now we react the sample with another combination of derivatization reagent called as o-phthalaldehyde and N-acetyl-L-cysteine. This adduct is highly reactive secondary amino acids also, it is easily available (17).

Selective determination of secondary amino acids by reverse phase HPLC

The method for the selective analysis of secondary amino acids was given by Stephan Einarsson in 1985.This method gave alternatives to be taken to combat the disadvantages of O-phtahlaldehyde and 2-mercaptoethanol. It is unable to react and produce derivatives with secondary amino acids.In this method, the derivatization is done in such a way that both primary and secondary amino acids are being reacted and are derivatized completely. It involves the reaction and derivatization of primary amino acids by using normal o-phthalaldehyde and 2-mercaptoethanol and after that, subsequent derivatization of secondary amino acids by 9-fluorinylmethyl chloroformate (FMOC). Both primary and secondary amino acids are analysed by using such an approach in a basic solution. The next step after the derivatization of secondary amino acids is the detection of primary amino acids in the same reaction matrix (18).

4. Alternative methods for the estimation of Amino Acids

Jinnaong ye amd Richard P Baldwin in 1994 gave another method for the simultaneous estimation of amino acids by another technique without the need of derivatization and the technique they used was capillary electrophoresis. This method involves the separation of amino acids by capillary electrophoresis and then the separated sample is electro-oxidized on the copper electrodes so that we can quantify the samples.The only condition of this process to work efficiently is that the electrode requires a completely alkaline ambience around it and that can be achieved by using around 50-100 mM sodium hydroxide (NaOH).The detection limit of amimo acids using this CE method was found to be around 1-10 fmols. One of the main advantages of using cu electrodes is that when we use electrodes other than that of copper, they require a pulse potential to operate, whereas, in the case of

copper electrode, no such condition exists and it can operate on a constant potential only (19).

Xuimei Jiang et al in 2009 devised another improvement in the estimation of amino acids by capillary electrophoresis (CE) technique. They developed an online sweeping technique for the estimation of amino acids simultaneously. With the help of this online sweeping technique, the LOD or limit of detection for the samples came to value of 0.1 to 0.5 µmol/L. This technique saved the time and efforts that were being consumed in other primitive techniques for the same purpose. The next step is the direct UV detection of the samples after separation. By using this method, till now, various real life samples of amino acids in the human urine, saliva and other secretions have been analysed. The direct UV detection can be done by using copper electrodes as the centre ion and this allows the detection at somewhat shorter wavelength of 254 nm, wavelength at which most of the amino acids are generally analysed (20).

Before the development done by Xuimei et al, Zhaohui et al in 2001 gave a novel method of capillary electrophoresis with laser induced fluorescence for the detection of the amino acid samples in the human brain. This could be done by derivaizing the amino acid samples by using a different agent other than the conventionally used ones, that is 5-flourylquinoline-3-carboxaldehyde .This technique is of special significance where the sample is of biological origin and the available amount of the sample is too less to be analysed by the usual analytical techniques. An argon ion laser was applied to the samples for the purpose of inducing fluorescence in the non-fluorescent samples which were not able to exhibit the phenomenon of fluorescence naturally. Subsequently, the derivatized amino acid samples were examined for the qualitative and quantitative analysis. This LIF (Laser Induced Fluorescence) technique was more advantageous over the other conventional techniques in various aspects like this technique enhanced the sensitivity of the analytical procedure hence bringing down the detection limits to a very less value. Another advantage that this technique serves is its very less sample demand. This requires a very less volume of the sample for an efficient analysis work. Hence this technique is less time consuming, inexpensive and precise (21).

5. Conclusion

This paper describes various analytical techniques available for the simultaneous estimation and analysis of amino acids. We discussed various methods developed by various researchers over time for the estimation of amino acids using the technique of High Performance Liquid Chromatography (HPLC). The comparison of the two available techniques of analysis by HPLC was also done and it was found that out of the two, post column and pre column derivatization, pre-column derivati-zatiom is considered to be more efficient and effective for the simultaneous estimation of amino acids. The pre-column technique gives a more accurate and precise result of the analysis as compared to the post column derivatization procedure and also it is less time consuming. Further we found that there are other available techniques also the estimation of amino acids than HPLC only. We can consider the technique of capillary electrophoresis (CE) for the estimation purpose. This technique gives the results without the help of derivatization. But in the end, it is the pre column derivatization, which would be of more use since it enhances the sensitivity and precision of the analytical work.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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