Amino acid analysis as their 9-fluorenylmethyloxycarbonyl-derivatives by HPLC

Theses of doctoral dissertation

Andrea Jámbor

Semmelweis University
Doctoral School of Pharmaceutical and Pharmacological Sciences

Tutor: Ibolya Molnár-Perl, D.Sc.

Opponents: Szilvia Bősze, Ph.D.
Mária Gazdag, Ph.D.

Head of Examination Committee: Imre Klebovich, D.Sc.
Members of Examination Committee: Ilona Török, Ph.D.
Miklós Józan, Ph.D.

Eötvös Lóránd University
Institute of Chemistry, Department of Analytical Chemistry
Budapest
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Introduction

The living organism consists of many N-containing compounds (certain neurotransmitters, porphyrins, polyamines, enzymes and nitric oxide), of which amino acids are precursors, in particular the protein building amino acids, the essential building blocks of life.

The malfunctioning of certain metabolic processes lead to change(s) in the amount of amino acids in the body tissues and cells. The change in the volumes and/or the ratio of the change(s) can go by different diseases, enzyme defects, such as phenylketonuria, maple syrup disease, metabolic disorders of homocystine, arginine, as well as the therapeutic efficacy of the developed drugs, analyzing the accordingly prepared cell cultures, tissues, body fluids.

Genes encoded amino acids, consequently the result of genetic engineering are shown by measuring amino acid. The genetic science is one of the most prevalent sciences in these days.

Amino acids are the building blocks of proteins, not only, but also serve as a source of energy. Thus, the concentrations of amino acids in dietary supplements and infusion solutions must be controlled.

Based on the above, it appears that developing a reliable analytical method for qualitative and quantitative determination of amino acids is crucial necessity for modern biochemistry, medicine, clinical diagnostics, taxonomy, genetic-, food-, chemical- and pharmaceutical sciences.

Aim and Scopes

My work aimed to develop a method for quantitative and qualitative determination of amino acids as their 9-fluorenylmethyloxycarbonyl (FMOC) derivatives. The controversial points of literature data have been critically evaluated. The reaction of amino acids and FMOC has been optimized as a function of:

- the pH values of borate buffer (pH 8, 9, 10),
- the solvent of FMOC reagent (acetone, acetonitrile),
- the concentration of FMOC (0.25 - 5 mM) and
- the molar ratio of [FMOC]/[amino acids]^{T}.
- The reaction time for the quantitative derivatization has been determined as a function of reaction conditions.
- The presence and amounts of impurities and the FMOC-OH have been taken into account and determined as a function of reaction conditions.
- The formation and transformation of Hystidine [*N*-FMOC-Hystidine (His-1) and *N,NH*-$(FMOC)_2$-Hystidine (His-2)] and Tyrosine [*N*-FMOC-Tyrosine (Tyr-1) and *N,O*-$(FMOC)_2$-Tyrosine (Tyr-2)] have been investigated with the quantitative evaluation of both derivatives.

The developed method has been applied: plasma free amino acids have been determined.

Methods

Samples

Serum samples have been obtained from the National Institute of Oncology, taken from females of breast cancer (ages 55-65), before surgery and 3 weeks after surgery. Patients have taken no oncological therapy. The blood samples have been centrifuged (4000 rpm, 4°C, 10 minutes), supernatant was stored in freezer. Samples have been used immediately after dissolving.

Standard solutions

Standard solutions of free amino acids have been prepared with distilled water at 0.03 M concentrations of each (weighed with analytical precision), stored in refrigerator and further diluted before use, in every second day.

Buffer solution

Borate buffer was mixed from 25 mL 0.8 M boric acid (dissolved in 0.8 M potassium chloride solution), adjusted with 0.8 M sodium hydroxide to pH 8.00, 9.00 and 10.00, thereafter diluted with distilled water up to 50 mL (further on buffer solution).

Reagent solutions

FMOC solution: the stock solution of FMOC was prepared with 0.026 g FMOC, weighed with analytical precision into 5.0 mL acetonitrile (20 mM) and further diluted before use (further on FMOC solution).

ADAM solution was prepared with 0.06 g ADAM.HCl, was weighed with analytical precision into 5 mL, acetonitrile/0.2 M HCl = 1:1 volume ratio (80 mM) and was used without dilution.

HEPA solution was prepared with 0.058 g heptylamine, diluted with distilled water (100 mM) and was used without dilution.

Characterization of reagent solutions

Blank runs were performed with reagent solutions every day at least twice. (Robotic Autosampler was thermostated at +20°C.)

Optimum derivatization and deproteinization conditions

In case of model solution: Derivatization following optimum conditions, were performed by mixing in the autosampler vial (1 mL; Waters, Milford, MA, USA), in order of listing 150 µL amino acids solutions (containing in total $5.4x10^{-8}$ mol amino acids in distilled water) with 150 µL buffer solution (0.4 M, pH 9) and with 300 µL FMOC reagent, dissolved in acetonitrile (containing $3x10^{-7}$ mol; $[FMOC]/[amino\ acids]^T = 5.5:1$). After 20 minutes the reaction was stopped by adding 50 µL ADAM or 50 µL HEPA solutions.

Serum samples have been prepared as above, with the following differences:

- they have been diluted (7x, 15x, 30x) and used with or without fortification by standard amino acids (containing $\leq 4x10^{-8}$ mol free amino acids);
- the amount of FMOC in the reaction mixture was 1.8 mol ($[FMOC]/[amino\ acid]^T = 47:1$);
- thereafter the solution was transferred through the cellulose cartridge (850 µL, pore size 0.45 µm; W. R. Grace & Co, Deerfield, Conn. USA) into the autosampler vial and centrifuged: 5000 rpm for 10 min (Centrifuge was Hungarian product: Zuglói Gépgyár, Budapest, Hungary).

Model studies, resulting optimum conditions, were carried out in the same reaction mixture volume of various compositions, detailed in the dissertation.

Chromatography

The HPLC system was a Waters HPLC (Waters Pharmaceutical Division, Milford, MA, USA), consisting of a Waters 996 DAD, Waters 274 fluorescence detectors, Waters 600 Controller Quaternary pump with controlled temperature column region, Waters 717 Autosampler, operating with Millennium software (version 2.10, 1992-95). Detection was made simultaneously, using the photodiode array (DAD, Waters 996) and fluorescence (FL, Waters 274) detectors, in that order. Blank and test samples were measured between 190 and 400 nm (DAD), evaluated at 262 nm (FMOC-amino acids).

The columns were (I) Hypersil-120 ODS (5 µm, 150 x 4.6 mm; BST, Budapest, Hungary) and (II) Thermo Hypersil-100 ODS (5 µm, 150 x 4.6 mm; Thermo Fisher Scientific Inc. Waltham, MA, USA). The guard columns were Hypersil ODS (5 µm, 30 x 4.6 mm; BST, Budapest, Hungary).

The conditions of separation: elutions were performed at 50°C, in gradient mode [the sodium acetate solution and distilled water were filtered (0.7 µm); degassed eluents were ensured by He] and gradient flow rate was used.

Four eluents were applied:

A: 0.05 M sodium acetate, pH 7.20;

B: 0.1 M sodium acetate/acetonitrile = 23:22 (v/v), pH 7.20;

C: 0.1 M sodium acetate/acetonitrile/tetrahydrofurane = 23:11:11 (v/v), pH 7.20;

D: acetonitrile/distilled water = 80:20 (v/v).

New scientific statements, conclusion

1. The quantitative interaction between the amino acids and the FMOC reagent(s) has been determined under various conditions: aspartic acid proved to be the rate limiting amino acid.

2. The optimum derivatization conditions were defined (borate buffer pH 9, acetonitrile containing 0.5 mM FMOC reagent, {[FMOC]/[amino acids]^T = 5.5:1, solvent/water = 1:1 (v/v)}, reaction time 20 min, reagent excess elimination by 1-aminoadamantane).

3. It has been proved that the reaction time of the quantitative derivatization is determined by the FMOC concentration, while,

4. on the basis of derivatizations, performed under various molar ratios of the reactants {[FMOC]/[amino acids]^T = 2.5:1 – 66:1}, it has been confirmed that the reaction rate could not been influenced by increasing these molar ratios. In addition, it turned out that in order to ensure the necessary FMOC excess the ratio of [FMOC]/[amino acids]^T should be $\geq$ 5.5:1.

5. Impurities, co eluting with the FMOC-amino acids, have been quantified for the first time. Based both on FL and on UV detections it has been demonstrated that to take into consideration the amount of these impurities is inevitable necessary.

6. As to the intrinsic phenomenon of the pH values of the derivatization reactions, it has been studied and defined for the first time. It was shown that pH values, given in the literature, are related to the aqueous mixture of the buffer and the amino acids (apparent pH values). After mixing with the FMOC reagent, immediately, a considerable change in pH values takes place and pH proved to be constant after any time (real pH

values). Under optimum derivatization conditions this increment of pH change proved to be 1.6 pH units.

7. The separation of 22 FMOC-amino acids was carried out by eluents of pH 7.2.

8. The formation and transformation of the single and two FMOC group containing derivatives of histidine (His-1, His-2) and tyrosine (Tyr-1, Tyr-2) have been quantitatively characterized.

9. The simultaneous deproteinization and derivatization method was introduced and applied to the quantitative determination of the plasma free amino acids.

Summary

In the frame of my studies the controversial points of literature data about the reaction conditions of amino acids and 9-fluorenylmethyloxycarbonyl-chloride (FMOC-Cl) have been critically evaluated. A reliable analytical method for qualitative and quantitative determination of amino acids as their FMOC-derivatives have been developed: the optimum conditions of the derivatization reaction were defined and the separation of 22 FMOC-amino acids was carried out. A new method was introduced for the determination of plasma free amino acids.

Publications

1. Á. Kőrös, R. Hanczkó, **A. Jámbor**, Y. Qian, A. Perl, I. Molnár-Perl: Analysis of amino acids and biogenic amines in biological tissues as their *o*-phthalaldehyde/ethanethiol/fluorenylmethyl chloroformate derivatives by high-performance liquid chromatography: A deproteinization study. *J Chromatogr A* (2007) 1149: 46-55.

2. R. Hanczkó, **A. Jámbor**, A. Perl, I. Molnár-Perl: Advances in the o-phthalaldehyde derivatizations: Comeback to the o-phthalaldehyde-ethanethiol reagent. *J Chromatogr A* (2007) 1163: 25-42.

3. **A. Jámbor**, I. Molnár-Perl: Amino acid analysis by high-performance liquid chromatography after derivatization with 9-fluorenylmethyl-oxycarbonyl chloride: Literature overview and further study. *J Chromatogr A* (2009) 1216: 3064-3077.

4. **A. Jámbor**, I. Molnár-Perl: Quantitation of amino acids in plasma by high performance liquid chromatography: Simultaneous deproteinization and derivatization with 9-fluorenylmethyloxycarbonyl chloride. *J Chromatogr A* (2009) 1216: 6218-6223.

Oral presentations

1. „Aminosavak Tanulmányozása Fluorenilmetil Kloroformáttal Történő Származékképzés Útján: Előkészítésük HPLC Analízisre"
   - oral presentation at Conference of "Scientific Days of Semmelweis University"
   Budapest, 12-13th April 2007

2. „Aminosavak Tanulmányozása Fluorenilmetil Kloroformáttal Történő Származékképzés Útján: Előkészítésük HPLC Analízisre II."
   - oral presentation at Conference of "Young Analysts' Seance"
   Budapest, 20th November 2007

3. „Aminosavak HPLC Analízise 9-fluorenilmetoxi-karbonil-számazékokként"
   - **oral presentation** at "Session for Working Committee of Organic and Pharmaceutical Analysis, Hungarian Academy of Sciences"
   Budapest, 18th May 2009

Poster presentations

1. „The Stability and Characteristics of the *o*-Phthalaldehyde Derivatives of Amino Acids and Amines"
   - poster presentation at Conference of "Pharmacy: Smart Molecules for Therapy"
   Budapest, 12-14th October 2005

2. „Analysis of Amino Acids and Biogenic Amines in Biological Tissues as Their *o*-Phthalaldehyde/Ethanethiol/Fluorenylmethyl Chloroformate Derivatives by HPLC: a Deproteinization Study"
- poster presentation at Conference of "ISC 2006"
Copenhagen, Denmark, 21-25th August 2006

3. „Behavior and Characteristics of the Fluorenylmethyl Chloroformate Derivatives of Amino Acids: Prepared for Their HPLC Analysis"
- poster presentation at Conference of "HPLC 2007"
Ghent, Belgium, 17-21st June 2007

4. „Behavior and Characteristics of the Fluorenylmethyl Chloroformate Derivatives of Amino Acids: Prepared for Their HPLC Analysis II."
- **poster presentation** at Conference of "HTC 10"
Bruges, Belgium, 30th January 2008 – 1st February 2008

5. „Behavior and Characteristics of the Fluorenylmethyl Chloroformate Derivatives of Amino Acids: Analysis of Biological Samples"
- **poster presentation** at Conference of "ISC 2008"
Munster, Germany, 20-25th September 2008

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