DERIVATIZATION OF AMINO ACIDS AND AMINES WITH *O*-PHTHALALDEHYDE REAGENTS: COMPARISON OF VARIOUS THIOL-CONTAINING PRODUCTS APPLYING HPLC

Theses of Doctoral Dissertation

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1. Introduction

Nitrogen plays a key role in the construction of the living organisms as the constituent of amino acids, proteins, hormones, coenzymes and as the building stone of terrestrial life also. The amino acids and certain amines are of great importance in physiological processes also Biogenic amines are not only part of our life as neurotransmitter but also as hormones that have an influence upon or modify the secretion of other hormones. It is assumed that their dysfunctional secretion and/or metabolism might be the reason of some diseases. The role of biogenic amines in the development of essential hypertony was widely examined. The histamine plays role inflammatory and allergic processes. Putrescine, cadaverine, agmatine and histamine are formed by decarboxylation of various amino acids.

Our research group extendedly examined the most optimal conditions of the derivatization, suitable for HPLC separation, of free amino acids and amines by *o*-phthalaldehyde (OPA) – in the presence of different thiols.

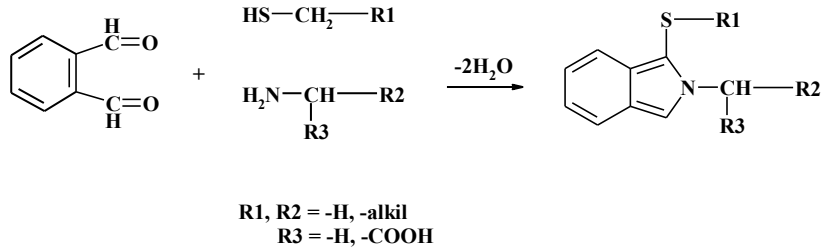

Figure 1.: Reaction of *o*-phthalaldehyde with primary amino group-containing compounds

The primary amino group-containing compounds form 1-alkylthio-2-isoindole (henceforth: isoindole) in alkaline medium, by reacting with OPA in the presence of an SH compound, being most often 2-mercaptoethanol, (MCE), 3-mercaptopropionic acid, N-acetyl-L-cysteine (NAC) or ethanethiol (ET). The advantages of this reaction are that

- it occurs in aqueous solution,
- within a short period of time.
- Removing the excess reagent is not necessary.
- Both UV and fluorescent detection may be applied.

The derivatization with OPA is very popular since its welcomed introduction in 1971. This is proven by the fact as well that during the period of 1992–1998, in 31.2 % of all the cases when amino acids were determined, OPA was applied. When reviewing the last five years' literature: 278 publications of almost 800 are found where amino acids or amines were OPA-derivatized, applying HPLC. It is also to be noted that the composition of the reagent applied was only in the case of

these 278 publications fully appropriate. In more than 60 % of these cases, MCE was used as SH-additive.

Our earlier and recent studies mostly focus on those primary amino group-containing compounds that form more than one derivative; this phenomena is being unexpected. Literature data and our experiences also showed that the quality of the thiol greatly determines the stability of the derivatives formed.

Following questions arise when revising the literature:

- What connection exists between the contradictory literature data about the stability of OPA-amino acids and the diverse reagent compositions?
- Do the OPA/MPA(NAC, MCE, ET) reagents have any self-fluorescence, that is to be eventually subtracted, when quantitating amino acids and amines while applying simultaneous UV and fluorescence detections?
- Within what period do OPA reagents provide equal response values?
- What is the cause, if there is any, that the derivatives of glycine, β-alanine, a γ-aminobutyric acid, histidine, ornithine and lysine are believed to be particularly less stable than those obtained with other amino acids?
- What fluorescent and UV response values provide the primary formed isoindoles, compared to each other, and are those values a function of the molar ratio of the OPA to the amino acid(s)?
- What is the structure and according to what mechanisms are the products formed from the primary isoindoles?

2. Aim of this work

I studied the reaction between the primary amino group and *o*-phthalaldehyde, applying 2-mercaptoethanol and ethanethiol as the SH-additive, while separating with high performance liquid chromatography, with simultaneous UV and fluorescent (and in some cases mass spectrometric) detections. Under strictly the same conditions,

- the stoichiometry of the reaction was studied in order to
- identify its mechanism and
- to utilise this knowledge for analytical purposes.

The conscious planning of the followings is considered as thoroughly identical conditions:

- the molar ratio of the *o*-phthalaldehyde to the SH-additive,
- and to the primary amino compound,
- the pH, the temperature and the duration of derivatization.

- The eluents and the type of the analytical columns, used for the HPLC separation - according to our possibilities – were the same as well.

I compared the obtained results both with the literature data and with the earlier results of our research group.

In order to be able to measure spermidine and spermine more sensitively, after applying the o-phthalaldehyde, the use of 9-florenylmethyl chloroformate as a second-step derivatization was introduced.

After the optimisation of both reactions and the conditions of the separation by HPLC, the amino acid and amine contents of biological tissues were measured. An HPLC method was elaborated, with which it is possible to determine the free (proteinogenic) amino acid and amine (being particularly interested in putrescine, cadaverine, spermidine and spermine) content of biological tissues. The simultaneous HPLC separation of 37 compounds in total was developed, applying two-step pre-column derivatization.

3. Methods applied

3.1. HPLC system (in order of listing):

„Waters 717plus" autosampler with thermostat; „Waters 600" ternary gradient pump and controller with temperature controlled column area, applying helium for the degassing of the eluents; „Waters 996" diode array detector (DAD) (scanning range: 190–410 nm); „Waters 474" scanning fluorescence detector. The system operates with the Millennium 2010 programme.

The detection of the OPA derivatives was carried out with DAD (at 334 nm), and fluorescence ($\lambda_{ex}/\lambda_{em}$=337 nm/454 nm) detectors, connected in order of listing..

3.2. On-line HPLC-MS system:

ThermoFinnigan TSQ Quantum AH instrument (ThermoFinnigan, LC-MS Division, San Jose, CA, USA): „Surveyor" diode array detector, „TSQ Quantum AH" (ElektroSpray-Ionisation, in the positive mode) MS detector, „Surveyor" autosampler. „Surveyor" ternary gradient controller with temperature controlled column area. The system operates with the Xcalibur 1.4 SRI programme. Mass range of the MS detector: 50–1600 mass units; The gas temperature was 200 °C (at a flow rate of 200 μl/min) or 380 °C (at a flow rate of 1 ml/min). The DAD was carried out at 334 nm. These measurements were carried out at the Chemistry Department of Central Service for Plant and Soil Protection, with the kind help of dr. Ferenc Tóth.

3.3. Columns:

The OPA/MCE derivatives were separated by using BST Hypersil ODS 150 mm×4 mm, 5 μm column (O-1). As a precolumn, 20 mm×4 mm, 5 μm BST Hypersil ODS (E-1) was used. The separation of the OPA/ET derivatives was commenced by using the columns O-1 and E-1, and continued by using a Thermo Hypersil ODS 200 mm×4.6 mm, 5 μm column (O-2). With a 30 mm×4 mm, 5 μm BST Hypersil ODS precolumn (E-2). {N.B.: The separation of the OPA/MCE derivatives of *n*-hexylamine, *n*-heptylamine, *n*-octylamine and *β*-phenylethylamine was carried out by using the columns O-2 and E-1.}

For the HPLC-MS measurements, columns O-1 and E-1 were used.

During the elaboration of the separation of the 37 compounds, the guard column E-1 was used. The main column (O-3) was a Waters X-Bridge C18 (150 mm×4,6 mm, 3,5 μm). This column can be utilised in the pH range of 1–12. The temperature applied might up to 80 °C when using eluents of lower pH, and it can only be used up to 45 °C when having eluents of high pH value.

3.4. Applied eluents

- Eluent A: 0.10 mol/dm^3 sodium acetate solution was two-fold diluted with distilled water. (0.05 mol/dm^3 sodium acetate solution).
- Eluent B: 0.10 mol/dm^3 sodium acetate solution, acetonitrile and methanol were mixed in the volume ratio of 46:44:10.

The eluents' pH was adjusted to 7.2±0.05 either with glacial acetic acid or 30 g/100 cm^3 sodium hydroxide solution added dropwise. Before use, the eluents were filtered through 0.45 μm Whatman filter paper, followed by ultrasonic degassing under vacuum. During the chromatographic separations, the eluents were constantly degassed with helium.

Methanol and/or acetonitrile were/was also applied in order to ensure a better separation.

3.5. Reagent solutions

The OPA/MCE and OPA/ET were prepared in a 10,00 cm^3 volumetric flask, by mixing 2,5 ml of OPA stock solution and

- 20 μl (molar ratio of OPA/MCE=1/3) or
- 340 μl of MCE (molar ratio of OPA/MCE=1/50), or
- 38 μl of ET (molar ratio of OPA/ET=1/10)

and by completing with 0.2 mol/dm^3 borate buffer of pH=9.3. Before that, the pH was adjusted to 9.3 if needed. If a reagent of different pH value was needed, a borate buffer of that pH was prepared also. The reagent obtained had a methanol content of 25 (V/V)%.

In the case of the OPA/ET reagent the methanol content was mostly 80 (V/V)%. This reagent was prepared in a 10.00 cm^3 volumetric flask by mixing the calculated amount of OPA stock solution, ET and 2.00 ml of 0.8 mol/dm^3 borate buffer of an appropriate pH value.

The FMOC solution was prepared from 0.17 g of FMOC-Cl, weighed with analytical precision and filled up to 5.00 cm^3 with acetonitrile.

The reagent solutions and the amino acid/amine solutions were mixed in a manner to ensure the molar ratio of OPA/(amino acid and/or amine)=20/1 and OPA/FMOC=1/0.4 in the reacting volume.

4. Summary; the novel results of the dissertation

1. Stability and the possibility of analytical application of the derivatives of amino acids, aliphatic mono- and diamines, formed with the *o*-phthalaldehyde (OPA)/2-mercaptoethanol (MCE) and OPA/ethanethiol (ET) reagents were examined. Products were detected by fluorescence and photodiode array detection, simultaneously. Applying strictly the same experimental {high performance liquid chromatographic (HPLC)} conditions, as the function of

–the reagents' composition,

–varying the molar ratio of OPA to thiol, the temperature and the duration of the reaction, was examined and characterized by quantitative data, based on stoichiometric investigations.

2. It has been proven that the stability of the derivatives, formed with – until recently – most often applied OPA/MCE reagent is considerably lower than those obtained with the OPA/ET reagent.

–The experimental conditions of the OPA/ET derivatives were optimised, lacking detailed literature data, as the function of the molar ratio of OPA to the thiol, the temperature of the elution, the pH value and methanol content of the reagent/reaction medium. Under the optimised conditions, quantitation reproducibility of amino acids and amines was measured in a wide range of concentration (3.43–440 pmol/compound injected) and characterised by the relative standard deviation percentages (RSD≤4.8 %, in average).

3. The behaviour and characteristics of the derivatives of *n*-hexyl-, *n*-heptyl-, *n*-octyl- and *β*-phenylethylamines, formed with OPA reagents of various thiol-content {MCE, ET, 3-mercaptopropionic acid (MPA), N-acetyl-L-cyisteine (NAC)}, lacking literature data, were described.

–It has been proven that the most advantageous HPLC separation and quantitation of these derivatives is achieved with the OPA/ET=1/10 reagent.

–Based on UV absorbency maxima values and mass spectrometric (MS) molecular ions, the by-product, containing two molecules of thiol, was identified qualitatively and quantitatively the first time by HPLC as a result of this study.

4. Detailed investigation of the OPA-derivatives of biogenic amines (histamine, agmatine, tyramine, putrescine, and cadaverine) amongst them the spermidine and spermine (containing secondary amino groups also) was carried out. It was proven that in the case of spermidine and particularly in the case of spermine, their measurement, with response values similar to those obtained with other amino acids/amines, is only made possible by derivatizing their secondary amino groups as well.
–Based on this recognition, the two-step (1^{st}: OPA/ET; 2^{nd}: 9-fluorenlymethyl chloroformate) derivatization of spermidine and spermine was described here the first time.
–Simultaneously, the OPA/ET/FMOC products were identified by HPLC-MS as well.

5. The procedure, elaborated for the analysis of biogenic amines, was utilised to determine the content of different biogenic amines of biological tissues. Large number of reproducibly analysed biological tissues of various quantities and the proportionality of the results to the weighed tissues do confirm the practical advantage of this method.

6. Extending the analysis described above, the quantitation of ornithine and lysine, simultaneously with the biogenic amines, was elaborated. Beyond the method's novelty, unexpected reaction of ornithine and lysine with the OPA/ET/FMOC reagent was experienced. It has been proven, that the reaction of ornithine and lysine with the OPA/ET/FMOC reagent yields derivatives of mixed ligands. This experience, verified by MS data, demonstrates a difference in the reactivity of the δ-/ε- and α-amino group of ornithine/lysine.

7. The simultaneous analysis of all the proteinogenic amino acids, aliphatic mono- and diamines, 37 compounds in total was studied. The qualitative and quantitative analysis, within 60 minutes, of all the 37 compounds was developed.
–It was proven that the 37 derivatives, mainly those eluting with a longer retention time, do decompose to an extent depending on the elution's temperature and on the pH of the eluents. Therefore the analysis of amino acids and amines is, more advantageous, to be preferred from separate elutions.

8. As a net result of this thesis, it is shown that

–the optimal conditions for the 'OPA-derivatization', being the most popular preparation for HPLC separations, were confirmed multi-facetedly.

–Thus in accord with and completing the earlier results of our research group, and hopefully contributing to the acceptance of the fact that the application of OPA/MCE-derivatization is the least advantageous solution.

5. Publications, posters, oral presentations

5.1. Publications:

-publications, already published:

(1) R. Hanczkó, I. Molnár-Perl: *„Derivatization, Stability and Chromatographic Behavior of o-Phthaldialdehyde Amino Acid and Amine Derivatives: o-Phthaldialdehyde/2-Mercaptoethanol Reagent"*, Chromatographia, Supplement Vol. 57 (2003) S-103–S-113.

(2) Hanczkó, D. Kutlán, F. Tóth, I. Molnár-Perl: *„Behavior and characteristics of the o-phthaldialdehyde derivatives of n-C6-C8 amines and Phenylethylamines with four additive SH-containing reagents",* R., J. Chromatogr. A, 1031 (2004) 51–66.

(3) R. Hanczkó, Á. Kőrös, F. Tóth, I. Molnár-Perl: *„Behavior and characteristics of biogenic amines, ornithine and lysine derivatized with the o-phthalaldehyde-ethanethiol-fluorenylmethyl chloroformate reagent"*, J. Chromatogr. A, 1087 (2005) 210–222.

-publications in preparation:

(4) R. Hanczkó, I. Molnár-Perl: *„Characteristics and stability of amino acids and C1-C5 aliphatic amines as their o-phthalaldehyde/ethanethiol derivatives"*, J. Chromatogr. A, in preparation

5.2. Posters and abstracts

- R. Hanczkó, I. Molnár-Perl: *Derivatization, Stability and Chromatographic Behavior of o-Phthaldialdehyde Amino Acid and Amine Derivatives: o-Phthaldialdehyde/2-Mercaptoethanol Reagent* (**poster**: 2002 – 24th International Symposium on Chromatography, Leipzig, Germany; Elválasztástudományi Vándorgyűlés, Lillafüred)
- Hanczkó R., Perlné Dr. Molnár I.: *Aminosavak és aminok o-ftálaldehid származékainak stabilitása és jellemzése: eltérő SH-csoportú segédanyagok jelenlétében* (**poster**: 2003 – Congressus Pharmaceuticus Hungaricus XII., Budapest)
- R. Hanczkó, D. Kutlán, Y. Mengerink, A. Csámpai, F. Tóth, T. Törő, I. Molnár-Perl: *The Role of the SH-Group Containing Additive in the Stability and Characteristics of the o-Phthalaldehyde Derivatives of Amino Acids and Amines* (**poster**: 2003 – 27th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Nice, France)

- R. Hanczkó, D. Kutlán, F. Tóth, I. Molnár-Perl: *Behavior and characteristics of the o-phthaldialdehyde derivatives of n-C6-C8 amines and Phenylethylamines with four additive SH-containing reagents* (**poster**: 2003 – 27th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Nice, France)
- R. Hanczkó, A. Perl, I. Molnár-Perl: *Optimum Conditions for the o-Phthalaldehyde Derivatization of the Primary Amino Group-containing Compounds in Biological Tissues* (**poster**: 2003 – 5th Balaton Symposium on High-Performance Separation Methods, Siófok)
- R. Hanczkó, I. Molnár-Perl: *Comparison of the Stability and Characteristics of the o-Phthalaldehyde/Ethanethiol and o-Phthalaldehyde/2-mercaptoethanol Derivatives of Amino Acids and Amines* (**abstract**: 2003 – 5th Balaton Symposium on High-Performance Separation Methods, Siófok)
- R. Hanczkó, A. Perl, I. Molnár-Perl: *Optimum Conditions for the o-Phthalaldehyde Derivatization of the Primary Amino Group-containing Compounds in Biological Tissues*, (**poster**: 2004 – 28th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Philadelphia, Pennsylvania, USA)
- R. Hanczkó, I. Molnár-Perl: *Derivatization, Stability and Chromatographic Behavior of o-Phthaldaldehyde Amino Acid and Amine Derivatives: o-Phthalaldehyde/Ethanethiol Reagent* (**abstract**: 25th International Symposium on Chromatography, Paris, France)
- R. Hanczkó, Á. Kőrös, I. Molnár-Perl: *Characteristics and stability of amino acids and C1-C5 aliphatic amines as their o-phthalaldehyde/ethanethiol derivatives* (**poster**: 2005 – 29th International Symposium on High Performance Liquid Phase Separations and Related Techniques, Stockholm, Sweden)

5.3. Oral presentations

- Hanczkó R., Perlné Dr. Molnár I.: *Primer aminocsoportot tartalmazó vegyületek o-ftálaldehiddel, 2-merkaptoetanol jelenlétében keletkező származékainak stabilitásvizsgálata: HPLC-vel, egyidejű ultraibolya és fluoreszcenciás detektálással* (2003 – OTDK, Budapest)
- Hanczkó R., Perlné Dr. Molnár I.: *Primer aminocsoportot tartalmazó vegyületek o-ftálaldehiddel, 2-merkaptoetanol és etántiol jelenlétében keletkező származékainak stabilitás-vizsgálata HPLC-vel, egyidejű ultraibolya és fluoreszcenciás detektálással* (2003 – Magyar Kémikusok Egyesülete, Fiatal Analitikusok Előadási Napja)
- R. Hanczkó, Á. Kőrös, F. Tóth, I. Molnár-Perl: *Behavior and characteristics of biogenic amines, ornithine and lysine derivatized with the o-phthalaldehyde-ethanethiol-fluorenylmethyl chloroformate reagent* (2004 – 25th International Symposium on Chromatography, Paris, France)