Journal of Chromatography A, 1390 (2015) 62–70

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Development of amino acid derivatization reagents for liquid chromatography electrospray ionization mass spectrometric analysis and ionization efficiency measurements

Riin Rebane*, Toomas Rodima, Agnes Kütt, Koit Herodes

Institute of Chemistry, University of Tartu, Ravila 14a, 50411 Tartu, Estonia

ARTICLE INFO

Article history:
Received 9 September 2014
Received in revised form 14 February 2015
Accepted 17 February 2015
Available online 22 February 2015

Keywords:
Amino acids
Derivatization
Matrix effects
LC-ESI-MS
Ionization efficiency

ABSTRACT

Derivatization is one of the most common ways for improving chromatographic separation and sensitivity for LC-ESI-MS analysis. The aim of this work was to design new derivatization reagents for LC-ESI-MS analysis of amino acids which would (1) provide good reversed phase chromatographic separation, (2) most importantly, provide low detection limits, (3) be easily synthesized, (4) produce derivatives which are less susceptible to matrix influences and (5) have convenient derivatization procedure with stable derivatives suitable for automatization. In the current work two new LC-ESI-MS compatible derivatization reagents have been designed and synthesized, dibenzyl ethoxymethylene malonate (DBEMM) and benzyl ethyl ethoxymethylene malonate (EBEMM). The DBEMM meets all the goals set with instrumental detection limits as low as 1 femtomole for amino acids and 40 attomole for selenoamino acids.

1. Introduction

Different instrumental analysis methods are used when sensitive analysis is targeted and liquid chromatography with mass spectrometric detection (LC-MS) is often used for these purposes, especially with electrospray ionization (ESI) source since it is suitable for wide range of analytes. However, for many compounds the ionization is not efficient enough for sensitive analysis, for example, amino acids [1]. For amino acid analysis with LC and ultraviolet detection (UV), derivatization has been used to improve sensitivity and chromatographic separation [2]. The same approach has been applied in the LC-ESI-MS since with derivatization it is possible to increase detection sensitivity and selectivity by means of MS/MS technique, to improve chromatographic retention or peak shape, to eliminate carryover, to facilitate sample cleanup, and to form a stable derivative for unstable analytes [3,4].

For mass spectrometric analysis of amino acids, two types of derivatization reagents are available. Firstly, derivatization reagents designed for ultraviolet or fluorescence detection such as dansyl chloride (DNS), 9-fluorenylmethyl chloroformate (FMOC-Cl), diethyl ethoxymethylenemalonate (DEEMM) [5] and 6-amino-quinolyl-N-hydroxysuccinimidyl carbamate (AQC) [6,7] can be used. These reagents are commercially available and are suitable for mass spectrometric detection if methods are carefully developed [1,5,8,9]. Most specifically, signal suppression due to the co-eluting substances from the sample matrix or from the derivatization procedure itself, must be under close investigation [10]. It has been shown that boric acid used as a buffer for the derivatization can cause signal suppression if ESI-MS detection is used [11,12]. Although quite often for LC-MS applications, these derivatization reagents have not been designed with MS detection in mind.

Therefore, in recent years, there has been a rapid growth in design and development of amino acid derivatization reagents that are specially designed for mass spectrometric applications since more sensitive analysis is demanded [13]. Reagents such as (5-N-succinimidoxy-5-oxopentyl)triphenylphosphonium bromide (SPTPP) [14,15], 3-aminopyridyl-N-hydroxysuccinimidyl carbamate (APDS) [16], N-hydroxysuccinimide ester of N-alkylnicotinic acid (C_n-NA-NHS) [17] and p-N,N,N-trimethylammonioanilyl N′-hydroxysuccinimidyl carbamate iodide (TAHS) [18], 2,5-dioxopyrrolidin-1-yl N-tri(pyrrolidino)phosphoranylideneamino carbamate (FOSF) [9] and some others have been reported. In case of FOSF [5], TAHS [18] and SPTPP [14] better ionization in ESI is achieved by introducing the permanent charge into the derivative, but as a result chromatographic separation in reversed phased columns is somewhat problematic. A better chromatographic separation is achieved with a charge carrying C_n-NA-NHS but with

* Corresponding author.
E-mail address: riin.rebane@ut.ee (R. Rebane).

http://dx.doi.org/10.1016/j.chroma.2015.02.050
0021-9673/

water content over 50% in the eluent, ESI ionization efficiency is somewhat hindered [17]. At this point none of these specially designed derivatization reagents are commercially available.

Since generally the aim is to have sensitive analysis, the main aspect to consider when choosing derivatization reagents, is the limit of detection (LoD) and limit of quantitation (LoQ). These values depend on the structure of the derivatization reagents as well as on the particular instrument and its configuration used for analysis. For example, for derivatization of amino acids with AQC, it has been reported that when two different models of mass spectrometric instruments were used, with one instrument the detection limits were 2–222 fmol whereas with a more advanced instrument the detection limits were 0.031–2.7 fmol [19]. Similarly, TAHS detection limits are said to be around 0.1 fmol in ref. [18] whereas they are said to be in the range 2–39 fmol in reference [19]. Therefore, results obtained with the same instrument are more informative and the comparison of DEEMM, TAHS, FMOC-Cl and DNS amino acid derivatives with the same instrument showed that DEEMM (26–384 fmol) and TAHS (22–117 fmol) provide much lower limits of quantitation than FMOC-Cl (164–3615 fmol) and DNS (55–3887 fmol) [5].

Design of new derivatization reagents consists of multiple aspects and the compound, which is efficiently ionized in ESI source, is likely to have low limits of quantitation. Therefore, the knowledge of how different structural features of molecules influence its ionization efficiency would be the base for designing novel LC-MS derivatization reagents. Suitable scale of relative ionization efficiencies has been created in our work-group [20,21]. The ionization efficiency (IE) of compound B_1 relative to compound B_2 is defined as ratio of respective response factors according to equation (1),

$$\mathrm{IE}(B_1/B_2) = \frac{R_1/C_1}{R_2/C_2} \quad (1)$$

where R denotes the MS response and C denotes the concentration of the respective compounds. It is convenient to express ionization efficiency in logarithmic units, i.e. logIE. Ionization efficiency scales have been created for ESI ionization via protonation [21] and via Na-adduct formation [22]. These studies conclude that most influential parameters in determining the ESI ionization efficiency are pK_a, molecular volume and ability to chelate the charge carrier (H^+ or Na^+).

Choosing the right derivatization reagent is very important and a good derivatization reagent will provide the maximum sensitivity, yield, stability of derivatized compounds and also better chromatographic separation for as many amino acids as possible. In addition, the derivatization reaction has to be quantitative and each amino acid, including secondary amino acids, should produce one single product that must be sufficiently stable for analysis. Stable derivatives also allow storing samples for sufficiently long period in case reanalysis is necessary. From the synthesis point of view, a good derivatization reagent is relatively cheap and easy to produce and stable as well. For compatibility with MS detection, two properties of a substance are especially relevant: good ionization and minimal susceptibility to matrix effects (e.g. minimal susceptibility to ionization suppression due to the presence of other substances).

In this article, two new derivatization reagents are designed and synthesized based on the considerations that would be the most suitable for the LC-ESI-MS analysis of amino acids.

2. Materials and methods

2.1. Chemicals and solvents

1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) (Fluka, Japan), ethyl orthoformate (Fluka), acetic anhydride (Sigma–Aldrich), zinc chloride (Sigma–Aldrich), dibenzyl malonate (Aldrich) and 2-mercaptoethanol (Sigma). Seleno-methylselenocysteine (Se-MeSeCys) was kindly donated by LGC Limited (United Kingdom) and selenomethionine (SeMet) purchased from Sigma.

All aqueous solutions were prepared with ultrapure water purified by Millipore Milli-Q Advantage A10 (Millipore).

0.03 M phosphate buffer with pH 2.12 was prepared by mixing 0.03 M orthophosphoric acid with 0.03 M dihydrogenphospate and pH was measured.

0.75 M borate buffer was prepared in deionized water and pH was set to 9.00 with concentrated sodium hydroxide solution.

0.56 M HFIP buffer for derivatization was in aqueous solution and pH was adjusted to 9.0 with concentrated sodium hydroxide solution.

2.2. Synthesis procedure

The synthesis of DBEMM and EBEMM was based on the synthesis of DEEMM [23,24]. Ethyl orthoformate (3.06 g), acetic anhydride (3.83 g), dibenzylmalonate (4.95 g) and anhydrous zinc chloride (6 mg) were mixed in two-necked round-bottom flask equipped with a fractionating column (Vigreux column) (Fig. 1a). The column was attached to a still head and condenser. The flask was immersed into a glycerol bath. A Teflon coated magnetic stirrer bar was added to the mixture and the mixture of reagents was stirred on the magnetic stirrer. The contents of the flask were heated to 110 °C; after 2.5 h, the temperature was raised to 122 °C and maintained at this temperature for 14 h. After that, firstly ethyl orthoformate (0.65 g) followed by acetic anhydride (0.97 g) were added to the mixture via syringe and the following temperature program was followed: 137 °C for 2 h and 155 °C for 2 h. Then the mixture was cooled to the room temperature.

2.3. Purification procedure of DBEMM and EBEMM

Purification of DBEMM and EBEMM involved multiple steps. In the first step, the mixture was distilled under reduced pressure. At temperature 105–110 °C (6 Torr), fractions of some byproducts were collected. The remaining mixture (not distilled) contained DBEMM, EBEMM and some byproducts, which were purified by flash chromatography, where at first, normal phase separation was applied (for removing remaining byproducts) and then reversed phase for separating DBEMM and EBEMM. For the normal phase chromatography, a Reveleris Silica 40 g column was used. A sample from the synthesis was diluted (1:1) with ethyl acetate. Approximately 1.5 mL of sample was injected onto the column. Gradient elution at flow rate 24 mL/min was used with hexane and ethyl acetate applying the following gradient: 0–1.5 min, 31% ethyl acetate; 1.5–18 min, 31–100% ethyl acetate. The fraction from 12 to 16 min was collected and dried under vacuum. This mixture contained DBEMM and EBEMM.

In order to separate DBEMM and EBEMM, a Reveleris C18 12 g column was used. Isocratic elution mode at flow rate of 30 mL/min was used with 0.1% aqueous formic acid and acetonitrile (40:60) as mobile phase. Fractions from 2 to 3.5 min were collected for EBEMM and 4–7 min for DBEMM. Fractions were dried under vacuum to dryness to obtain pure substances.

2.4. Preparation of standard solutions

Stock solutions of individual amino acids (1–20 mg/g) and Se-MeSeCys and SeMet (0.55 mg/g) were prepared in 0.1 M hydrochloric acid with 30% methanol. Stock solution of SeMet (0.4 mg/g) was prepared in 0.5% 2-mercaptoethanol aqueous solution to prevent oxidation [8]. Stock solutions containing multiple amino acids were prepared once and stored at −20 °C. All dilutions