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Optimization of separation and detection of 6-aminoquinolyl derivatives of amino acids by using reversed-phase liquid chromatography with on line UV, fluorescence and electrochemical detection

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Abstract

The combined use of UV-absorbance, fluorescence and electrochemical detection was proposed for the analysis of a set of thirteen amino acids by reversed-phase liquid chromatography (RP-HPLC) using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate as a precolumn derivatization reagent. The utility of using three detectors in series was demonstrated. The separation of all derivatized amino acids was optimized with the aid of a computer optimization program from only four simple linear gradient measurements. The effectiveness of a reliable retention prediction of solutes under any gradient profile using other gradient or isocratic data was also examined.

Keywords: Amino acids; Reversed-phase liquid chromatography (RP-HPLC); 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC); Separation optimization; Ultraviolet–florescence–electrochemical (UV–FL–EC) detection

1. Introduction

Amino acid analysis is one of the fundamental measurements of biological science. The increasing popularity of reversed-phase HPLC methods comprising pre-column derivatization over the classical (ion-exchange) post-column techniques is probably due to the enhanced sensitivity, the less cost and perceived ease of use. Among many pre-column derivatizing reagents used for amino acid analyses 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) has become popular because of its simple derivatization procedure, the strong fluorescent intensity and stability of the derivatives as well as its commercial availability by Waters. AQC was initially introduced by Cohen and Michaud as a specific fluorescent derivatizing reagent for analyzing amino acids, which reacts with primary and secondary amino acids to yield derivatives with a fluorescent response and no significant interference from the only major fluorescent by-product, 6-aminoquinoline (AMQ) [1]. Additionally, UV-absorbance detection instead of florescence (FL) was proposed for the determination of AQC-derivatized amino acids [2] as well as an alternative electrochemical (EC) detection method [3], provided that the huge reagent peak does not interfere with the isolation of amino acid derivatives. However, up to now there is a luck of using UV, FL and EC detectors on line for improving amino-acid detectability in a variety of samples analyzed by the AQC-derivatization method [1–13].

As concerned the separation of AQC-amino acid derivatives, in most of the relative publications [2,4–10], it was performed on a Waters AccQ.Tag amino acid analysis Nova-Pak column following the original gradient conditions described by Waters [14] or some similar ones, empirically modified. However, it should be useful if gradient conditions for an optimum resolution of solutes were selected with the aid of a computer optimization program, especially when the separation of a complex mixture of AQC-derivatized amino acids is carried out with a column different than the specific one, AccQ.Tag column, proposed by Waters.

Thus, the main objective of this work is to improve the analysis of amino acids using a pre-column derivatization with AQC followed by RP-HPLC separation. For this purpose, instead of one detection mode and gradient conditions empirically established, we used three detectors in series and the gradient

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conditions were selected by means of a computer optimization program. This approach was applied to a mixture of 13 amino acids, which were previously analysed directly in their underivatized form [15–17], in order to be also feasible a comparison between the detection sensitivity and the retention behaviour of free amino acids and their derivatives with AQC.

2. Experimental

The solutes were the following 13 AQC derivatives of amino acids: L-histidine (his), L-carnosine (car), L-cysteine (cys), *S*-methyl-L-cysteine (me-cys), DL-homocysteine (hcy), 5-hydroxy-tryptophan (5htp), L-tyrosine (tyr), L-methionine (met), DL-m-tyrosine (m-tyr), DL-alpha-methyltyrosine (me-tyr), 3-nitro-L-tyrosine (n-tyr), L-phenylanine (phe) and L-tryptophan (trp). The derivatization reagent AQC was obtained as a kit from Waters Corporation (Milford, MA, USA). The kit named Waters AccQ.Fluor Reagent Kit contains reagent power (AQC), reagent diluent acetonitrile (MeCN) and 0.2 mM sodium buffer, pH 8.8. AQC derivatization was done by following the kit instruction sheet and the procedure optimized by Cohen and Michaud [1]. An aqueous amino acid standard mixture was prepared at a concentration of 10 μg mL^{-1} for each amino acid. For derivatization 10 μL of this mixture of standards were buffered to pH 8.8 with the borate buffer to a total volume of 80 μL. The derivatization was initiated by addition of 20 μL of 10 mM AQC in MeCN and then the derivatized solution was heated to 55 °C for 10 min. Thus the final concentration of each amino acid derivative in the analyzed mixture was 1 μg mL^{-1}.

The liquid chromatography system consisted of a Shimadzu LC-20AD pump, a model 7125 syringe loading sample injector fitted with a 20 μL loop, a 250 mm × 4.6 mm MZ-Analysentechnik column (Perfectsil 120 ODS 5 μm), a Shimadzu dual UV–vis spectrophotometric detector (Model SPD-10A) with a flow cell volume of 8 μL, a Shimadzu spectrofuorometric detector RF-10AXL with a cell capacity of 12 μL and a Lab-made multiple electrochemical detector equipped with a glassy carbon electrode (3 mm diameter). The UV, FL and EC detectors were connected in series so that the analytes separated on the HPLC column flowed through the UV detector first, then through the FL detector and at the end through the EC system. This allows a multiple measurement of analytes by UV absorbance at 254 nm, FL detection at $\lambda_{ex}/\lambda_{em}$ = 248/395 nm and oxidation response at 1.1 V versus the Ag/AgCl reference electrode. The EC detector maintained at 1.1 V, except for generating hydrodynamic voltammograms. Hydrodynamic voltammograms were constructed by obtaining multiple chromatograms of a given sample at 100 mV increments with the applied potential varying from 0.5 to 1.3 V.

Mobile phases consisting of a 0.02 M aqueous phosphate buffer with pH 2.5 and MeCN as organic modifier were used. All experiments were carried out at 25 °C using a Shimadzu CTO-10ASVP column oven. The flow rate was 1.0 mL min^{-1}, whereas the dwell time, i.e. the time needed for a certain change in the mixer to reach the beginning of the column, t_D, and the hold-up time, t_0, were estimated as 1.1 and 2.43 min, respectively. Only retention times of the solutes obtained by the UV detector were used to the separation optimization procedure.

3. Results and discussion

3.1. Separation optimization of AQC-amino acid derivatives

In order to optimize the separation of a mixture of solutes, such as the 13 AQC derivatives of amino acids adopted in the present study, the first step is to determine the dependence of the solute retention factor, $k_\varphi = (t_\varphi - t_0)/t_0$, upon the mobile phase composition, φ, where φ is the volume fraction of the organic modifier in the water–organic mobile phase, t_φ is the isocratic retention time of the solute at a mobile phase concentration equal to φ and t_0 is the column hold up time. In this investigation we used the following expression for the dependence of k_φ on φ [18]:

$$\ln k_\varphi = a - \frac{c\varphi}{1 + b\varphi} \quad (1)$$

where a, b, c parameters are treated as adjustable parameters that may be determined from either isocratic or gradient data.

The determination of parameters a, b, c of Eq. (1) from either isocratic or gradient data was carried out by a home-made modification of the Levenberg–Marquardt (LM) algorithm presented in a recent paper [15] and named RND-LM algorithm. Note that the fitting process of gradient data to Eq. (1) proposed in Ref. [15] presumes the analytical expressions derived in that paper for the calculation of the retention time t_R of a sample solute under multilinear gradient conditions However, that approach appeared a drawback, which limited considerably the flexibility of the treatment in practical applications [15]. For this reason, in the present study, t_R under any gradient profile was calculated by means of the stepwise gradient elution theory [16,19] since, according to Cela et al. [20–25], any practical programmed solvent gradient can be made equivalent to a stepped gradient formed by just 11 equispaced proportions obtained by dividing the total modifier range into 10 equal segments. In our study for more accurate results each gradient profile was approximated by a stepwise gradient consisting of 100 equidistant steps.

Thus the retention behavior of AQC-derivatives was studied under both isocratic and gradient conditions. The isocratic retention times of the derivatives under consideration are depicted in Table 1. The values of the a, b, c fitting parameters of Eq. (1), calculated by the RND-LM algorithm from the above isocratic data, with their standard deviations and the standard error of fit, s_y, are listed in Table 2. The fitting of Eq. (1) to gradient data was based on data obtained under four gradient profiles shown in Fig. 1 by numbers 1, 2, 3 and 4. The experimental retention times of amino acids derivatives under these gradient profiles together with the predicted ones by the a, b, c values of Table 2, following a procedure described bellow, and the percentage error of the prediction are given in Table 3. The experimental data of Table 3 were fitted to Eq. (1) by means of the RND-LM algorithm but the values of the a, b, c parameters determined showed great uncertainties, which means that every time the algorithm ran,