Derivatization of amino acids in a moving zone of
o-phthalaldehyde in the middle of a capillary for amino acid
analysis by capillary electrophoresis

Atsushi Taga, Mayumi Sugimura, Susumu Honda*

Faculty of Pharmaceutical Sciences, Kinki University, 3-4-1 Kowakae, Higashi-Osaka 577, Japan

Abstract

Problems in in-situ derivatization in a narrow zone in the middle of a capillary was investigated using a model system composed of amino acids and *o*-phthalaldehyde (OPA). Hydrostatic introduction of a solution of amino acid(s),the running buffer and the OPA solution for specified periods of time in this order from the anodic end of a capillary, followed by application of an appropriate voltage between both ends, resulted in formation of the OPA derivatives of amino acids, which were immediately separated by zone electrophoresis while moving through the rest of the capillary and detected at the detector window. Peak intensity was varied among amino acids, due to the variation of zone-overlapping period, accordingly reaction time. This technique of in-capillary derivatization was liable to be affected by various factors, including sample/reagent introduction times and applied voltage, giving rather lower reproducibility of determination than the derivatization at the inlet. However, it was useful for kinetic studies of such rapid reactions as those adopted in this work as a model system.   © 1998 Elsevier Science B.V.

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1. Introduction

The recent progress of capillary electrophoresis (CE) is overwhelming and this method has become utilized more and more in various fields of life science. The major advantages of this method are its high capabilities in separation and detection, since it allows theoretical plate numbers as high as several hundred thousands and sensitive detection at levels as low as amol. CE has other advantages inherent to this method. For example, it easily allows analysis in free solution, which is equivalent to a pseudochromatographic technique in liquid chromatography. The ease of free solution analysis permits observation of physical interaction between two substances based on migration delay of one of the substances introduced into a buffer containing the other, as demonstrated by our previous paper on carbohydrate–protein interaction [1]. Free solution analysis also allows in-situ chemical derivatization in a buffer filled in a capillary. We have also been studying this technique.

Since CE is usually performed in free solution in a narrow capillary, a special section of a capillary can be reserved for chemical reaction. If such section is secured at the inlet of a capillary, components of a sample can be derivatized by overlaying a sample and reagent solutions, followed by standing the plugs for an adequate period. The derivatives thus produced can be analyzed immediately by applying a voltage. We have already published a paper on the derivatization of amino acids with *o*-phthalaldehyde

*Corresponding author.

(OPA) at the inlet of a capillary and confirmed the reliability of this technique for their quantification [2]. This technique of derivatization seems generally useful for analysis of substances difficultly detectable due to lack in chromophore or fluorophore, and especially promising for automated analysis of biological and environmental substances. Gilman and Ewing [3] successfully applied this method to the analysis of dopamine and several amino acids in a single mammalian cell using naphthalene-2,3-dicarboxaldehyde and CN^- as the derivatization agents. The derivatives formed at the capillary inlet were separated by zone electrophoresis and sensitively detected at the detection window by argon laser-induced fluorescence. Zhou et al. [4] reported continuous on-line derivatization of aspartate and glutamate in microdialysis perfusate of rats with the same derivatization agents. In this case the reaction took place in a small reactor placed before the capillary inlet, from which a small portion of derivatized perfusate was introduced repeatedly, at appropriate intervals, to the capillary for a short period by switching a sampling valve. Therefore, this technique was not at-inlet derivatization, though similar to it.

The special section of a capillary is not necessarily confined to the inlet. We have extended the position of the section for chemical reaction to other parts of a capillary. In this paper we describe model experiments, in which reaction occurs in a narrow zone in the middle of a capillary. Regnier and coworkers [5,6] studied enzyme reactions throughout a capillary. This work is valuable, because it gives important information for activity assay and mechanism elucidation. We have studied application of this throughout-capillary format to not only enzyme reaction but also reaction of small molecules. However, we would like to discuss such kinds of work separately from the present paper, since we regard this format as the third one of in-capillary chemical derivatization. Reyderman and Stavchansky [7] reported on-line intercalation of single-stranded oligodeoxy-nucleotides with the OliGreen reagent for laser-induced fluorescence detection, but this is different from the zone-passing format of the in-capillary derivatization technique we describe in this paper, because intercalation occurred just before the detection window at an extremely high concentration of the dye in this case. Intercalation is not chemical

reaction, but physical interaction. Observation of physical interaction occurring in a capillary [8,9] is also important, because it gives useful information to kinetic studies under conditions similar to physiological environment using small amounts of biological substances. However, it should be discussed apart from in-capillary derivatization.

Post-capillary derivatization [10,11] resembles the throughout-capillary format of in-capillary derivatization, but there is a fundamental difference between these two formats in that the former is performed after separation, whereas the latter prior to or concurrently with separation. Therefore, post-capillary derivatization should be discussed separately from the throughout-capillary format of in-capillary derivatization.

2. Experimental

2.1. Apparatus

CE was performed using a Jasco 890-CE high-voltage power supply and a Jasco 875-CE UV–Vis detector. A capillary (50 μm I.D.) of fused-silica was obtained from Polymicro Technologies (Phoenix, AZ, USA) and a 70-cm portion was used throughout the work. It was flushed with 0.1 M sodium hydroxide before use and equilibrated with the running buffer (50 mM borate buffer, pH 10.0) prior to each run by using an aspirator. In the in-capillary derivatization experiments the sample and reagent solutions, as well as the running buffer, were introduced hydrostatically by raising the levels of these solutions, in which the anodic end of the capillary was immersed, by 10 cm higher than the level of the cathodic solution for specified periods of time.

2.2. Chemicals

A reagent grade sample of OPA was purchased from Wako (Osaka, Japan) and used without purification. Other chemicals and amino acid specimens were of the highest grade commercially available. The sample solutions were prepared by dissolving either an amino acid or a mixture of selected amino acids in the running buffer. In the optimization experiments the concentration of each amino acid

was fixed at 2.5 mM, but in the preparation of the calibration curves eight points were selected for each amino acid concentration at 0.1, 0.5, 1.0, 3.0, 4.0, 5.0 mM. The reagent (OPA) was used as a 50 mM solution in the running buffer.

2.3. Derivatization in a capillary

A sample solution, the running buffer and the reagent solution were introduced to the anodic end of the capillary in this order for specified periods of time, and a specified voltage was applied between both ends of the capillary. The peak of the product was monitored at 230 nm.

3. Results and discussion

3.1. Strategy for derivatization in the middle of a capillary

Derivatization in a zone in the middle of a capillary can be achieved by successive introduction of a sample solution, the running buffer and the reagent solution, followed by application of a voltage, as illustrated in Fig. 1.

If the reagent zone moves faster than the sample zone, the former passes over the latter during migra-

tion. Reaction occurs while both zones are overlapping each other in the middle of a capillary. If the reagent zone moves slower than the sample zone, the introduction order of the sample and reagent solutions should be reversed. Furthermore, if the sample and reagent zones move in the opposite directions, they must be introduced from the different ends of a capillary.

3.2. Electropherograms

In the model system employed a sample solution of selected amino acids, the running buffer, and the reagent solution were successively introduced from the anodic end in this order by hydrostatic introduction. Finally running buffer was introduced again for a short period to prevent the reagent solution from flowing back to the reservoir. This is only for precaution of possible slight pressure decrease at the inlet. In this system an alkaline buffer was used, hence electroosmotic flow was rapid toward the cathode and the amino acids were drawn back more strongly than the reagent by electromigration. Therefore, the reagent zone migrated faster than the sample zone toward the cathode by the total effect of electroosmosis and electrostatic force, and caught up and passed the sample zone in the middle of a capillary. During the passing process the sample
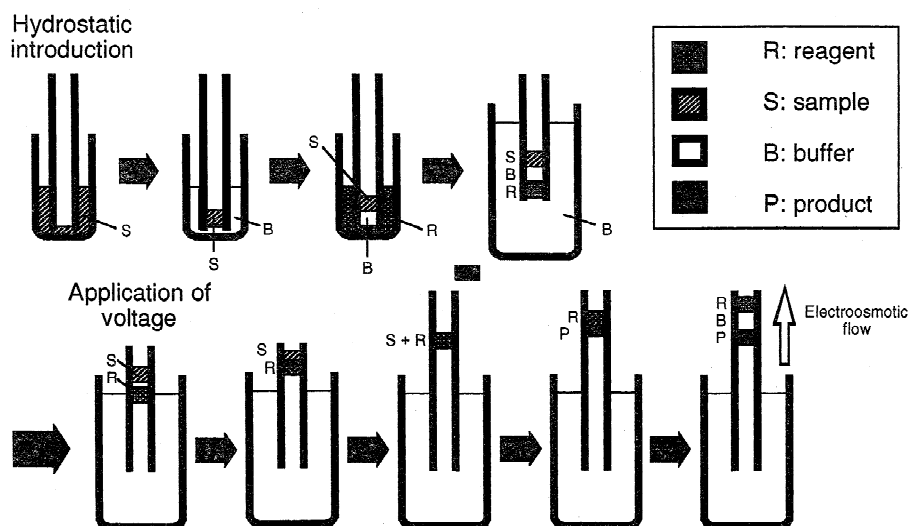

Fig. 1. Sample/reagent introduction scheme for in-capillary derivatization in a zone in the middle of a capillary.
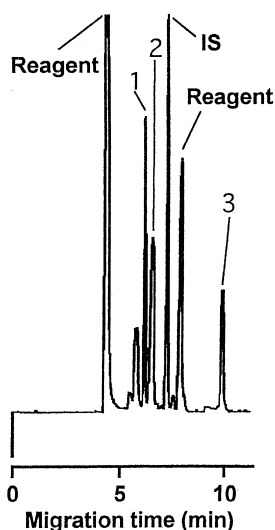
Fig. 2. Electropherogram of OPA derivatives of selected amino acids formed by derivatization in a zone-passing mode in the middle of a capillary. The derivatization and analysis were performed by successive introduction of a sample solution, the running buffer and the reagent solution for 10, 10 and 60 s, resepectively, followed by application of 20 kV to both ends of the capillary. Peaks: 1=L-methionine, 2=L-alanine, 3=L-glutamic acid, I.S.=internal standard (cinnamic acid). Other analytical conditions are described in Section 2.

reacted with the reagent to give OPA derivatives of amino acids (OPA–amino acids), which moved at their own velocities faster than the sample amino acids and slower than the reagent. As a result an electropherogram as shown in Fig. 2 was obtained.

In the derivatization at the inlet of a capillary by the reagent/sample/reagent sandwich introduction mode [2], the excess reagent gave a twin-headed peak due to incomplete mixing [1], whereas the derivatization in the present system did not give such a twin-headed peak. Migration profile was similar to that obtained by derivatization at the inlet. However, the peak intensity was divergent, mainly because of the variation in passing period, accordingly the variation in reaction time among amino acids.

3.3. Effects of various factors

3.3.1. Sample/reagent introduction time

Variation of peak response was also seen much more clearly in another experiment using only phenylalanine as sample (Fig. 3).

In this experiment the introduction times of the sample solution and the running buffer were maintained constant, but the introduction time of the
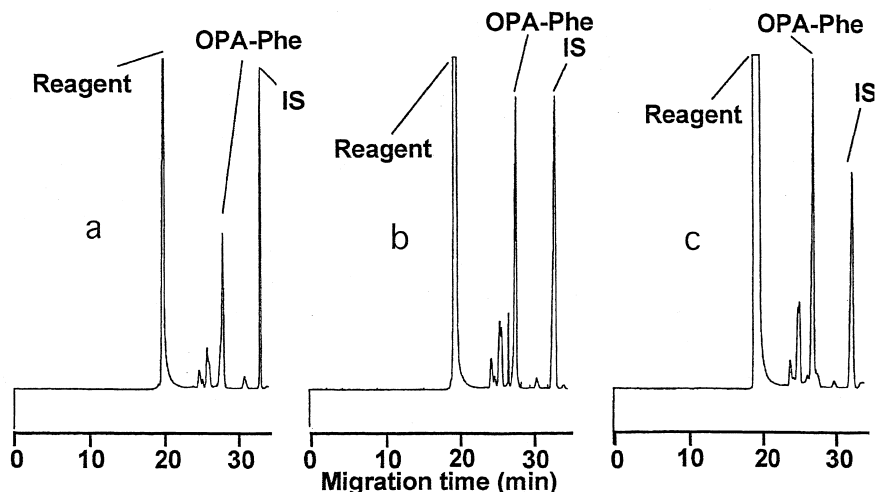

Fig. 3. Effect of reagent introduction time on the yield of OPA–phenylalanine in derivatization in a zone-passing mode in the middle of a capillary. Reagent introductin time (s): (a) 10; (b) 40; (c) 120. Applied voltage, 5 kV. Other conditions for derivatization and analysis as in Fig. 2. The attenuation in (a) and (b) is twice that in (c).

reagent solution was varied from 10 s to 120 s. Since the internal standard (cinnamic acid) was added to the sample solution, the introduced amounts of the sample and the internal standard were constant. The relative peak response of OPA–phenylalanine to the internal standard (cinnamic acid) increased, as the introduction time of the reagent solution increased as shown in Fig. 3, because the passing period increased.

We further observed the effect of changing the introduction time of the sample solution instead of the reagent solution. The graphs on the left-hand side (Fig. 4a) compare the relative peak response between the two introduction modes.

The introduction time of the sample was fixed and the introduction time of the reagent was varied in the lower curve, and vice versa in the upper curve. It is obvious that prolongation of reagent introduction time caused increase of peak response, whereas change of sample introduction time gave almost unchanged relative peak response. This is presumably because the sample concentration in the overlapping zone was much higher than the reagent concentration, and the derivatization reaction proceeded very quickly.

3.3.2. Applied voltage

Change of applied voltage also gave information on the derivatization. When the introduction times for the sample and the reagent were kept constant and applied voltage was increased, a descending curve was observed as in Fig. 4b. Increase of applied voltage decreased passing period, and thereby reduced relative peak response.

These results indicate that derivatization in the middle of a capillary is liable to be affected by various factors, including sample/reagent introduction times, their relative concentration, applied voltage, etc.

3.4. Calibration curves and reproducibility of determination

The calibration curves of amino acids under the optimized conditions (introduction time: sample solution, 10 s; running buffer, 10 s; reagent solution, 60 s) were linear (alanine: $y=0.181x-0.65\cdot10^{-3}$, $R=0.994$; methionine: $y=0.126x+1.79\cdot10^{-2}$, $R=0.999$; glutamic acid: $y=0.0897x-1.16\cdot10^{-3}$, $R=0.991$; where y and x are relative response to 1.0 mM cinnamic acid as the internal standard and molar
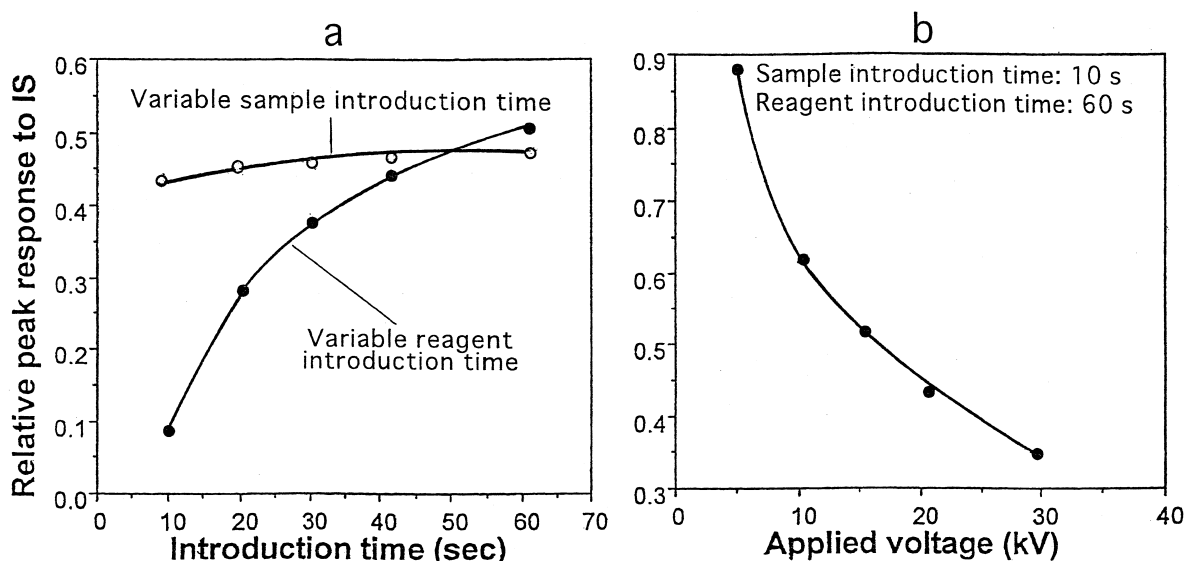
Fig. 4. Effects of sample/reagent introduction time (a) and applied voltage (b) on the yield of OPA–phenylalanine. Analytical conditions as in Fig. 3.

concentration, respectively) at least over the range 0.5~5 m*M*. However, the reproducibility of peak response was not so high as that in the derivatization at the inlet of a capillary; relative standard deviation ($n = 10$) exceeded 3.5% in some examples.

3.5. Estimation of the rate constant of the derivatization reaction

The zone-passing mode in the middle of a capillary mentioned above gave a useful information concerning reaction rate even for such rapid derivatization as observed here with OPA. Fig. 5 shows the plot of the ln value of amino acid (phenylalanine) concentration vs. reaction time.

Good linearity was observed, and the rate constant could be estimated from the slope of this line. The

reaction time in the abscissa could be estimated as the passing period, that is, the ratio of the sample plug length to migration velocity difference of the sample and the reagent. Sample plug length is approximately the reagent peak width at half height $W_{1/2}$, multiplied by $A^{-1}lt^{-1}$, where A, l and t are chart speed, capillary length between the inlet and the detector window, and the migration time of OPA, respectively. The velocity difference of the sample and reagent can be calculated as $l \cdot (t_{OPA}^{-1} - t_{Phe}^{-1})$, where t_{Phe} is the migration time of L-phenylalanine. From the slope of this line the pesudo first-order rate constant was estimated to be $8.83 \cdot 10^{-3}$ s^{-1}.

Thus, it was demonstrated that observation of the zone-passing mode of in-capillary derivatization gave useful information on the estimation of the rate constant of the derivatization reaction of amino acids with OPA. This technique will be generally useful for kinetic studies of chemical reactions, especially rapid reactions, and will be used widely in physical chemistry.
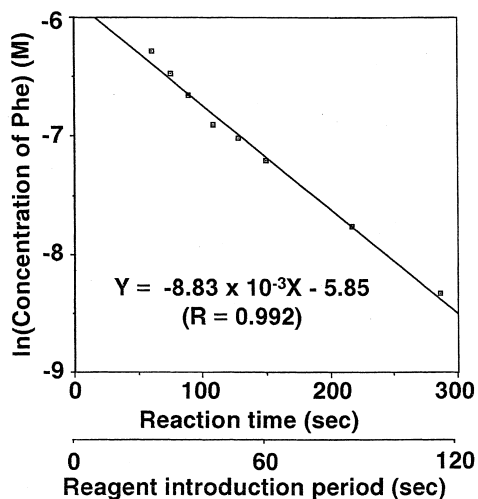

Fig. 5. Rate constant determination of the derivatization reaction of L-phenylalanine with OPA by the plot of ln (concentration of L-phenylalanine) vs. reaction time. The reaction time was calculated as the ratio of the length of the OPA plug (estimated as $W_{1/2}Alt_{OPA}^{-1}$, where $W_{1/2}$, A, l and t_{OPA} are the reagent peak width at half height, chart speed, capillary length between the inlet and detection window, and the migration time of OPA, respectively) in the variable reagent introduction time mode (in Section 3.3.1) to its velocity difference between L-phenylalanine and OPA–phenylalanine ($t_{OPA}^{-1} - t_{Phe}^{-1}$).

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