ELSEVIER

Journal of Chromatography A, 822 (1998) 271–279

JOURNAL OF CHROMATOGRAPHY A

Characteristic features of the throughout-capillary technique of in-capillary derivatization in capillary electrophoresis

Atsushi Taga, Aki Nishino, Susumu Honda*

Faculty of Pharmaceutical Sciences, Kinki University, 3-4-1 Kowakae, Higashi-Osaka 577-8502, Japan

Received 20 March 1998; received in revised form 11 August 1998; accepted 11 August 1998

Abstract

Characteristic features of the throughout-capillary technique of in-capillary derivatization for capillary electrophoretic analysis were explored using a rapid derivatization model. Selected amino acids (phenylalanine, glycine and glutamic acid) were converted to their OPA derivatives, while they were moving through an electrophoretic solution containing OPA in the electric field, and the resultant OPA–amino acids were concurrently analyzed by zone electrophoresis with UV detection. A deep trough appeared based on sample–reagent displacement, and the base line was noisy and drifty, especially at high reagent concentrations. Peaks were fronting and peak width varied among amino acid species, presumably due to the variation of the difference in velocity between an amino acid and its OPA derivative. However, the throughout-capillary technique is the simplest of all techniques of pre-capillary and in-capillary derivatizations, and there was good linearity between relative peak area and amino acid concentration. The quantification was reproducible with R.S.D.~3.5%. The rate constant of the derivatization reaction could be roughly estimated by plotting logarithm of amino acid concentration vs. reaction time. The obtained values were approximately identical with the values obtained by the zone-passing technique. This paper also compares this technique with other techniques of in-capillary derivatization with respect to peak area, signal-to-noise ratio and column efficiency.

Keywords: In-capillary derivatization; Derivatization, electrophoresis; Amino acids, OPA derivatives

1. Introduction

Capillary electrophoresis (CE) is noted for its high column efficiency and reproducible quantification by on-column detection. It has additional advantages over other existing separation methods. One of them is that it allows free solution analysis in an open tubular capillary. One can regard a part or the whole of the inner space of a capillary as a place where chemical reaction occurs, and thus can realize chemical derivatization in a capillary either prior to or concurrently with electrophoretic analysis.

In-capillary derivatization can be achieved by one of the following three techniques depending on where and how derivatization reaction occurs. The first one is the at-inlet technique, in which a sample and a reagent solutions are introduced to the inlet of a capillary either by tandem or sandwich mode, and these reactants are mixed by diffusion and allowed to react by standing the successive plugs for a specified period of time. The preliminary report by Tjaden et al. [1] is considered to be the first example of this technique and the patent by Fischman et al. [2] also

*Corresponding author.

0021-9673/98/$ – see front matter
PII: S0021-9673(98)00665-7

deals with it. Gilman and Ewing [3] reported a paper on single cell analysis, in which neurotransmittal substances in a cell were derivatized to fluorescent products at the inlet of a capillary following cell lysis. We examined quantification by this technique and applied it to automated analysis of amino acids as *o*-phthalaldehyde (OPA) derivatives [4].

The second technique, the zone-passing, is based on derivatization in the middle of a capillary by passing either a sample or a reagent zone through the other in the electric field. The order and site of sample/reagent introduction must be determined based on the magnitude and sign of the mobility, and peak intensity varies with passing period of time. This technique is useful for kinetic studies of rapid reactions [5].

The third approach is the throughout-capillary technique. Regnier and co-workers [6,7] developed a methodology designated EMMA for kinetic studies of slow enzyme reactions, using this technique. Oguri et al. [8] described derivatization of some amino acids with OPA. These authors reported mainly on quantification with fluorimetric detection, but neither discussed the basic features of this technique in detail nor compared it with other techniques of in-capillary derivatization. In the throughout-capillary technique an amino acid introduced from the inlet of a capillary continues to travel through an electrophoretic solution containing the reagent, OPA. Although the OPA derivative fluoresces strongly, neither unreacted amino acid nor OPA fluoresces, hence is not detected. Therefore, this system gives information on only the yield of the derivative. It is difficult to observe how the reactants mix and react with each other. Detection by UV absorption will give much more information on this technique of in-capillary derivatization, though it is less sensitive.

The present paper aims at revealing unknown aspects of the throughout-capillary technique and comparing this technique with other techniques of pre-capillary and in-capillary derivatizations.

2. Experimental

2.1. Instrumentation

High-performance capillary electrophoresis (HPCE) was performed by assembling a HEL30PI high-voltage power supply from Matsusada Precision Devices (Kusatsu, Shiga Prefecture, Japan) and an 870 UV–Vis detector from Jasco (Hachioji, Tokyo, Japan). In each technique of derivatization a capillary of fused-silica (70 cm×50 μm I.D.) from Polymicro Technologies (Phoenix, AZ, USA) was used. A detection window was made at the 50-cm position from the anodic end by removing a 500-μm portion of polyimide coating by burning. The capillary was flushed with 100 m*M* sodium hydroxide and equilibrated with the electrophoretic solution to be used, before each run. In pre-capillary derivatization and all techniques of in-capillary derivatization, detection was performed by monitoring UV absorption at 230 nm.

2.2. Chemicals

A reagent grade sample of OPA was obtained from Sigma (St. Louis, MO, USA). The samples of amino acids and cinnamic acid (CA) were of analytical grade commercially available. Electrophoretic solutions were made in deionized and glassware-distilled water and degassed before use.

Sample solutions were prepared by mixing an aqueous solution containing selected amino acid(s) at various concentrations together with CA (1.0 m*M*, otherwise stated) as an internal standard and 100 m*M* borate buffer, pH 10.0, in an equal volume. Reagent solutions were made by mixing an aqueous OPA solution at various concentrations and 100 m*M* borate buffer, pH 10.0, in an equal volume. Both of the sample and reagent solutions were prepared fresh before use.

2.3. Procedures for derivatization/electrophoretic analysis

2.3.1. Throughout-capillary technique

A capillary was filled with a reagent solution, and a solution of selected amino acid(s) was hydrodynamically introduced to a capillary by maintaining the sample solution level higher than the anodic solution level by 10 cm for 10 s. A potential of 20 kV was applied between both ends of the capillary, to move the amino acid(s) through the reagent solution

for derivatization and to transport the resultant OPA derivatives to the detector.

2.3.2. Other techniques (for reference)

In pre-capillary derivatization, amino acids were reacted with OPA in a reaction vial by a slight modification of the method of Roth [9], briefly as follows. A sample solution (20 μl) of three selected amino acids (L-phenylalanine, Phe; glycine, Gly; L-glutamic acid, Glu, 1.0 m*M* each) in 50 m*M* borate buffer, pH 10.0 was mixed with a 50 m*M* OPA solution (20 μl) in the same buffer, and the mixture was allowed to stand for 20 min at room temperature. The reaction solution was introduced to the anodic end of the capillary and analyzed immediately by HPCE using 50 m*M* borate buffer, pH 10.0 as running buffer.

In-capillary derivatization by the at-inlet technique was performed according to our previous paper [4]. Briefly, a solution of selected amino acids (0.5 m*M* each) and a 50 m*M* OPA solution each in 50 m*M* borate buffer, pH 10.0 were introduced successively to the anodic end of the capillary using the R(3 s)–S(1.5 s)–R(3 s) program of the sandwich mode with the vacuum injector system of an Applied Biosystems apparatus, where R and S are reagent and sample solutions, respectively, and the resultant succession of plugs of the sample and reagent solutions were allowed to stand for 20 min. During this period the zones were mixed with each other by diffusion and the samples were derivatized with OPA. The resultant derivatives were immediately analyzed by HPCE in the same running buffer as in pre-capillary derivatization by applying a voltage of 20 kV.

In-capillary derivatization by the zone-passing technique was according to our previous paper [5]. Briefly, a sample solution of selected amino acids (0.5 m*M* each) in 50 m*M* borate buffer, pH 10.0 was introduced for 1.5 s to the anodic end of the capillary by suction. After subsequent suction of the same running buffer as used for pre-capillary derivatization for 1.5 s, a reagent solution in the same buffer was introduced for 10 s. Upon application of a potential of 20 kV between both ends of the capillary, the reagent zone passed the sample zone. The amino acids reacted with OPA during the passing period. The resultant derivatives were transported to the detector.

2.4. Determination of the reaction rate constants

A 5.0 m*M* solution of an amino acid was introduced to an electrophoretic solution containing OPA to a concentration of 50 m*M*. The peak of the OPA–amino acid was taken to a Power Macintosh computer by use of a scanner, and the whole and fractional areas (WA and FA, respectively, in Fig. 5) were measured by desktop operation using a Flexi Trace 1.03J software (Three Stars, Sakuragaoka-cho, Shibuya-ku, Tokyo). The rate constant was obtained as the slope of the plot of log(amino acid concentration) vs. reaction time.

3. Results and discussion

3.1. Electropherograms

Derivatization of amino acids with OPA was adopted in the present work as a model system of fast reaction.

Fig. 1a shows the electropherogram of glycine derivatized with OPA by the throughout-capillary technique. Glycine moved faster toward the cathode than OPA–glycine under the conditions employed. OPA derivatives of amino acids not only fluoresce strongly but also absorb the UV light (λ_{max} 230 nm). The fluorimetric method can detect only the OPA derivatives, whereas the photometric method allows detection of not only the OPA derivatives but also OPA. A deep trough seen at ca. 4 min is the most characteristic of this derivatization technique, not observed with fluorimetric detection. This is considered to be due to the displacement of OPA in the electrophoretic solution (the reagent solution filled in the capillary) by glycine in the sample solution, which has weaker absorptivity than OPA. The main peak of OPA–Gly at ca. 6 min is broad and fronting, as compared to that in pre-capillary derivatization (Fig. 1b), presumably due to faster movement of glycine than OPA–Gly. There are also a few minor peaks around the main peak presumably due to by-products. The electropherogram of phenylalanine obtained under the same conditions gave a similar profile to that of glycine. Although the sample itself also absorbed the UV light in this case, it caused neither change of base line nor appearance of an extra peak. This is probably because the reaction rate

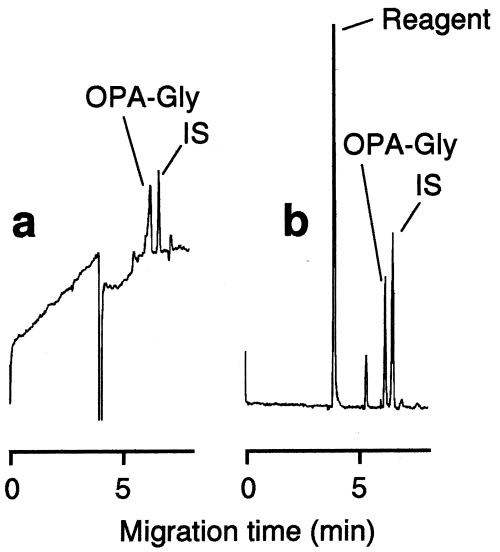

Fig. 1. Comparison of the analyses of glycine by the throughout-capillary technique of in-capillary derivatization (a) and pre-capillary derivatization (b). Capillary, fused-silica (70 cm×50 μm I.D.); electrophoretic solution (reagent solution), 50 m*M* borate buffer (pH 10.0) containing OPA (50 m*M*) (a) or not containing OPA (b); applied voltage, 20 kV; detection, UV absorption at 230 nm. The ordinate is corrected by the absorbance (ca. 0.28) of the baseline. The details for the sample and reagent solutions are described in Section 2.2. The concentrations of glycine and cinnamic acid (I.S.) were commonly 0.50 m*M*.

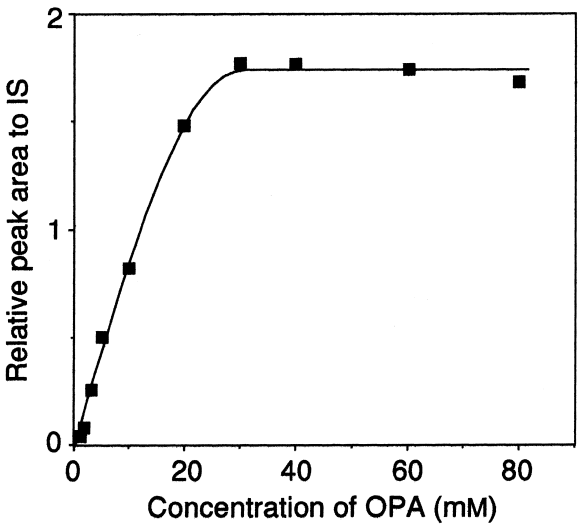

Fig. 2. The effect of OPA concentration on the relative peak area of OPA–Gly. Concentrations: glycine, 5.0 m*M*; cinnamic acid (I.S.), 3.0 m*M*.

of phenylalanine with OPA was so high that phenylalanine disappeared rapidly from the mixed zone giving no phenylalanine peak.

The intensity of the main peak rapidly increased with increase of OPA concentration in the electrophoretic solution and almost unchanged between 30 m*M* and 80 m*M* (Fig. 2).

Increased OPA concentrations higher than 100 m*M*, however, caused much noisy and sometimes drifty baseline (data not shown). The migration times of these peaks became shorter as applied voltage increased, but 20 kV was close to the upper limit due to excessively high electric current. At lower voltages the peak intensity was not different from that at 20 kV, indicating sufficient reaction time for derivatization.

Fig. 3 shows the electropherogram of a mixture of three selected amino acids derivatized with 50 m*M* OPA which is in the middle of the concentration

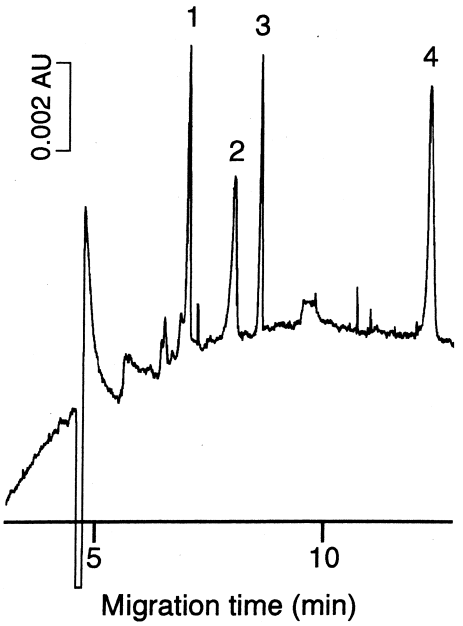

Fig. 3. Analysis of a mixture of the selected amino acids as OPA derivatives by the throughout-capillary technique of in-capillary derivatization. The concentration of each amino acid was 0.50 m*M*. Analytical conditions as in Fig. 1a. Peaks: 1=phenylalanine, 2=glycine, 3=cinnamic acid (internal standard), 4=glutamic acid.

range giving an absorption plateau. The time scale is double expanded from Fig. 1.

The derivatives of glycine and glutamic acid gave broad, fronting peaks having values of symmetry factor (the ratio of the half width of the whole peak to that of its front half divided by the perpendicular line from the peak top, at the 5%-position of peak height) around 0.8, whereas the peak of cinnamic acid as an internal standard gave quite a symmetric peak. In contrast phenylalanine gave an unexpectedly sharp peak. The variation of peak width can be attributed to the variation of velocity difference between an amino acid and its derivative. This was confirmed by actually estimating the velocity differences for these amino acids. Since the underivatized amino acids were not detectable at 230 nm, they were monitored at 200 nm using high-concentration solutions. The obtained values were as follows: phenylalanine, 0.88 cm/min; glycine, 2.3 cm/min, glutamic acid, 1.4 cm/min. The amino acids having small velocity differences, such as phenylalanine, will give sharp peaks, because the amino acids as raw materials move with nearly the same pace as their derivatives. On the contrary, if the amino acids move much faster than their derivatives, the peaks will be widespread and fronting, since the sample zone is always ahead of the derivative zone and the derivatization continues to occur in this preceding sample zone. Thus, glycine having the largest velocity difference gave the most wide and fronting peak. Although peak shape varied, all peaks of these three amino acids were well separated from each other under the conditions employed.

3.2. Quantification

Plot of relative peak area vs. amino acid concentration gave straight lines for all these amino acids examined ($y=0.454x+0.0575$, $R=0.996$ for Phe; $y=0.425x-0.0136$, $R=0.996$ for Gly; $y=0.659x-0.0468$, $R=0.999$ for Glu, where x and y are the amino acid concentration in m*M* and the relative peak area to 3.0 m*M* CA, respectively), at least over the 0.2–10.0 m*M* range.

The relative standard deviation (R.S.D.) of relative peak area at the 5.0 m*M* concentration was less than 3.5% (Phe, 2.6%; Gly, 3.3%; Glu, 2.8%). These values are slightly higher than the values obtained by the at-inlet technique (2.2–2.7%) [4].

3.3. Determination of reaction rate constants

Peaks in an electropherogram give useful information on the reaction rate of derivatization for individual amino acids. Plot of the proportion of the frontal triangle part (FA) of an observed peak (Fig. 5) vs. reaction time (τ) gave a concave curve, and the lnFA–τ plot gave a descending straight line. Since FA was proportional to the concentration of the remaining amino acid [r-AA], plot of ln[r-AA] vs. τ also gave a descending line. For example, the plot of ln[r-Phe] vs. τ gave a straight line expressed by $\ln[\text{r-Phe}]=-3.6\cdot10^{-2}\tau-6.99$ with a coefficient of correlation of 0.98, at the phenylalanine concentration of 5.0 m*M* (Fig. 4).

The concentration of the remaining phenylalanine concentration, [r-Phe], was estimated as 5.0 FA/WA m*M*. Thus, the reaction of phenylalanine with OPA was approximately of the first-order with phenylalanine concentration with a rate constant of $3.6\cdot10^{-2}$ s^{-1}. The reliability of this method depends on the accuracy of the measurement of FA for specified times. In the above estimation it was achieved by projecting the peak on the desktop of a computer by

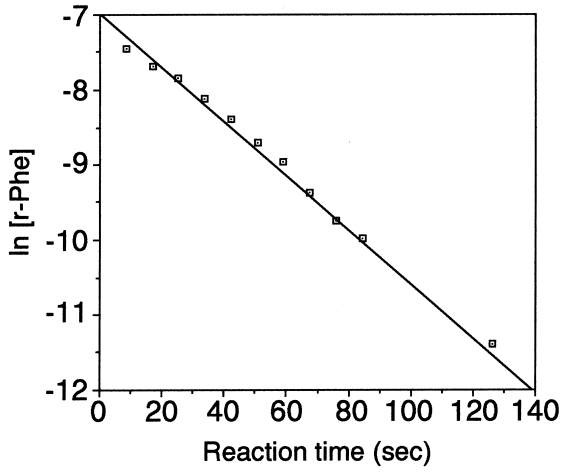

Fig. 4. Relationship between ln[r-Phe] and τ. The concentration of the remaining phenylalanine, [r-Phe], was obtained as 5.0 FA/WA. The value of reaction time τ, was estimated as described in Section 3.3.

use of a scanner and by measuring the areas of the frontal right-angle triangle underneath the peak at every additional 0.6 s, using a commercial software for area measurement. The peak obtained by introducing a solution containing phenylalanine alone to an OPA-containing electrophoretic solution (Fig. 5) was subjected to this treatment, because the OPA–Phe peak obtained for the ternary system (Fig. 3) was contaminated by a minor peak of an impurity.

The time interval of 0.6 s is corresponding to about 2.5% of the peak width, accordingly the time required for the derivative zone to pass the detector window. The value of τ was estimated as follows. In this derivatization Phe moved through the OPA-containing buffer and the derivatization was completed in the reaction time τ. The resultant OPA–Phe further moves to the detector window with a velocity different from that of Phe. Therefore, there is the following relationship among τ and the velocities of Phe (V_{phe}) and OPA–Phe ($V_{OPA-Phe}$),

$$V_{Phe}\tau + V_{OPA-Phe}(t - \tau) = \iota \quad (1)$$

where t and ι are the time when the resultant OPA–Phe is observed at the detector window and the effective capillary lengthy, respectively. Since V_{Phe} and $V_{OPA-Phe}$ can be expressed as ιt_{Phe}^{-1} and $\iota t_{OPA-Phe}^{-1}$, respectively, by using migration times of Phe and OPA–Phe, τ can be written by Eq. (2).

$$\tau = t_{Phe}(t_{OPA-Phe} - t)(t_{OPA-Phe} - t_{Phe})^{-1} \quad (2)$$

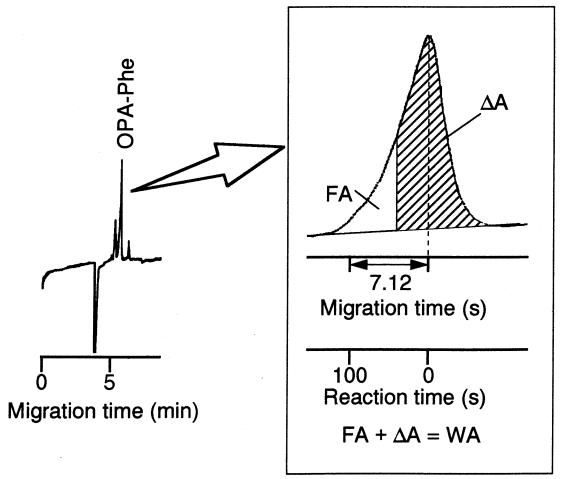

Fig. 5. Analysis of phenylalanine by the throughout-capillary technique of in-capillary derivatization for the determination of the reaction rate constant. The inset shows the relationship between FA and WA of an OPA–Phe peak. It also relates the τ-scale to the t-scale. Analytical conditions as in Fig. 1a.

For example reaction time (τ) of 100 s was obtained for the t value of 7.12, as illustrated in Fig. 5. Although such semi-manual treatment was performed carefully by using the special software, the estimation of the rate constant was not so accurate; use of a fully automatic integrator will give more reliable values of FA. The rate constants of glycine and glutamic acid with OPA determined in the same manner were $4.4 \cdot 10^{-2}$ s^{-1} and $5.1 \cdot 10^{-2}$ s^{-1}, respectively, though they are only approximate, because the correction of τ was not so reliable from the following reason. Accurate measurement of migration velocities of these amino acids was not easy, since their peaks could not be detected under the conditions employed. The migration velocity of each amino acid was estimated from a duplicate experiment by use of the same capillary and electrophoretic solution, but detected at a shorter wavelength (200 nm) using much higher concentrations of amino acids. The peaks were barely recognized due to noisy baseline.

3.4. Comparison with pre-capillary derivatization and other techniques of in-capillary derivatization

The foregoing results were compared to the results obtained by pre-capillary derivatization and other techniques of in-capillary derivatization using the same reagent and the same sample solutions under the conditions optimized for each. Fig. 6a shows the electropherogram of selected amino acids derivatized with OPA by pre-capillary derivatization. Fig. 6b and c show the electropherograms obtained by the at-inlet and the zone-passing techniques of in-capillary derivatization, respectively.

The pre-capillary derivatization gave sharp, symmetrical peaks for all amino acids and peaks were well separated from each other (Fig. 6a). The at-inlet technique of in-capillary derivatization also gave sharp, symmetrical and well-resolved peaks (Fig. 6b). The zone-passing technique gave a broadened peak of the main product for each amino acid, and peak height varied greatly among amino

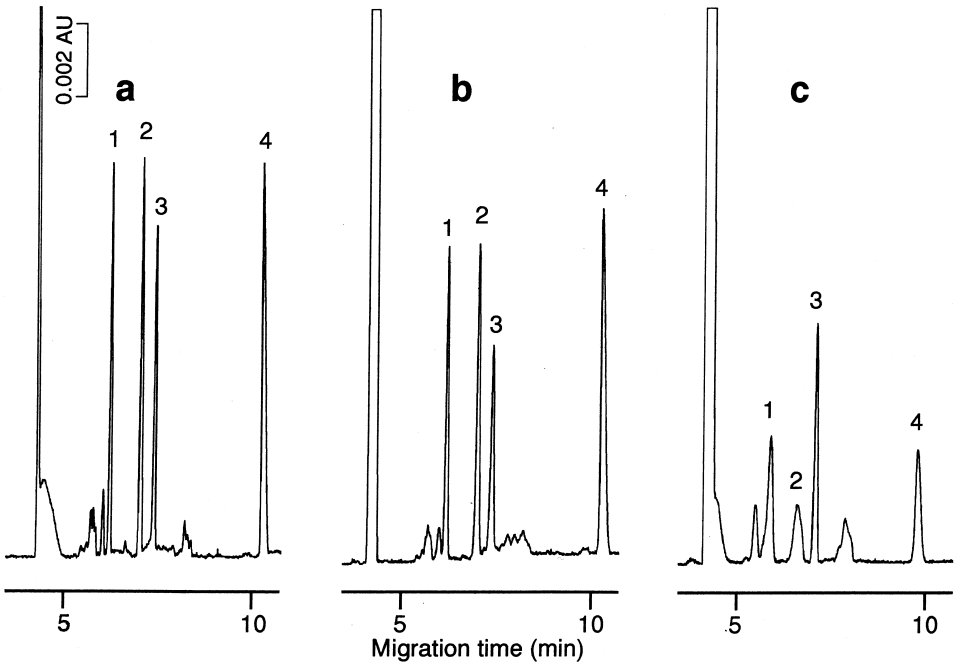

Fig. 6. Comparison of the analyses of selected amino acids by various methods and techniques of derivatization. (a) Pre-capillary derivatization; (b) and (c), the at-inlet and zone-passing techniques of in-capillary derivatization, respectively. Analytical conditions and peak assignment as in Fig. 3. The amino acid concentrations in the reaction mixture in (a) were the same as those in the zones in other techniques.

acid species [5], obviously due to the variation of the velocity difference between the amino acid and its derivative (Fig. 6c). It is observed that the order of peak height in the throughout-capillary technique in Fig. 3 (Gly<Phe<Glu) was the same as that in the zone-passing technique (Fig. 6c), again reflecting the order of the reactivity of these amino acids to OPA.

For further discussion three parameters, peak area, signal-to-noise (S/N) ratio and theoretical plate number, were calculated based on these electropherograms.

Fig. 7 compares peak areas. Pre-capillary derivatization and the at-inlet technique of in-capillary derivatization gave similarly higher values for all amino acids. The throughout-capillary technique gave the next high values and the zone-passing technique gave the lowest values. The peak area reflects completeness of reaction. The lowest area in the zone-passing technique is obviously due to shortage of reaction time.

Fig. 8 compares the signal-to-noise ratios. Pre-capillary derivatization and the at-inlet technique of

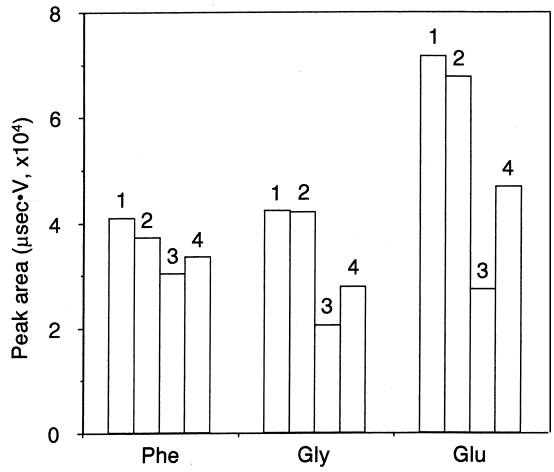

Fig. 7. Comparison of peak areas for the selected amino acids among various methods and techniques of derivatization. The data are based on the electropherograms in Figs. 3 and 6. Bar 1=pre-capillary; bar 2=at-inlet; bar 3=zone-passing; bar 4=throughout-capillary.

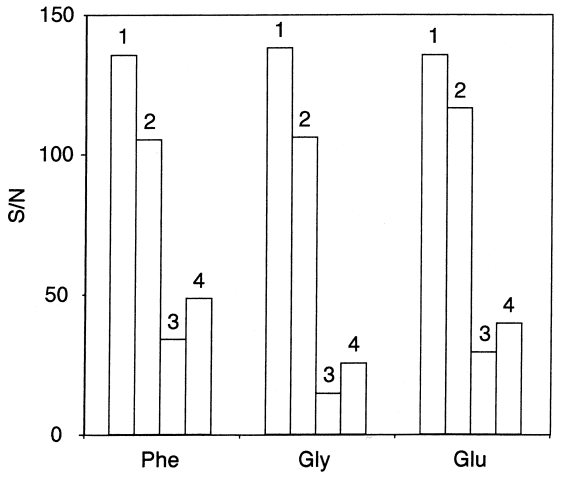

Fig. 8. Comparison of signal-to-noise ratios for the selected amino acids among various methods and techniques of derivatization. The data are based on the electropherograms in Figs. 3 and 6. Bars 1–4 as in Fig. 7.

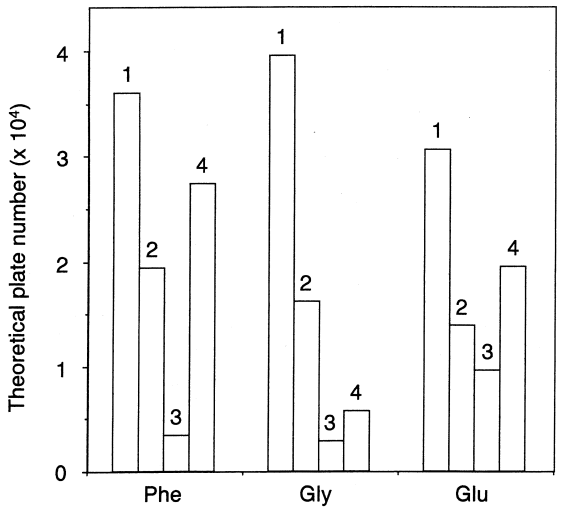

Fig. 9. Comparison of theoretical plate numbers for the selected amino acids among various methods and techniques of derivatization. The data are based on the electropherograms in Figs. 3 and 6. Bars 1–4 as in Fig. 7.

in-capillary derivatization gave high values again, whereas the zone-passing and throughout-capillary techniques gave low values. In these techniques, especially in the throughout-capillary technique, the baseline level was high due to the absorption of the UV light by OPA in the electrophoretic solution and peaks were buried in this high level base line.

Fig. 9 compares the theoretical plate numbers. It is reasonable that the pre-capillary derivatization gave the highest numbers for all amino acids, since derivatization proceeded completely outside the capillary and there was no reasons to cause broadening of the sample zone in the capillary except for molecular diffusion during electrophoretic migration. The at-inlet technique of in-capillary derivatization gave lower values due to additional zone broadening in the sandwich pile of reagent–sample–reagent plugs in the derivatization process. The zone-passing technique will give theoretical plate numbers depending on the introduction times of sample and reagent solutions, and it gave the lowest values under the conditions used. With regard to phenylalanine and glutamic acid the throughout-capillary technique gave higher values than the at-inlet technique. Sine pre-separational broadening does not exist in the throughout-capillary technique, peaks in this technique will be sharper than in the at-inlet technique, provided the velocity difference between the amino acid and its OPA derivative is small. The larger theoretical plate numbers in phenylalanine and glutamic acid mentioned above is considered to be an example of such case.

4. Conclusions

Amino acids could be converted to OPA derivatives, while they were moving through an electrophoretic solution containing OPA in the electric field, and the derivatives were concurrently separated from each other and detected by UV absorption at the detector window. This is the simplest technique of in-capillary derivatization, but there were some limitations. The electropherograms showed a tendency to give noisy and sometimes drifty baseline, especially at high reagent concentrations. There was a deep trough, which is inherent to this technique. This is because the reagent was positive to the detection method used (UV absorption method). Fluorescence detection, though not used in the present work, may eliminate the problem of appearance of such nuisance. However, monitoring of UV absorption gave such useful information as men-

tioned above to understand how the reactants were mixed and the reaction occurred.

Comparison of the peak areas and the S/N ratios among various techniques of in-capillary derivatization indicated the following priority: zone-passing < throughout-capillary < at-inlet. Theoretical plate number was generally in the order of zone-passing < at-inlet < throughout-capillary < pre-capillary, but it may vary depending on operating conditions. The migration order of theoretical plate number for the at-inlet and the throughout-capillary (at-inlet < throughout-capillary) techniques observed for phenylalanine and glutamic acid was reversed for glycine, due to large velocity difference between glycine and OPA–Gly.

Conclusively the throughout-capillary technique, which can be performed by the simplest operation, allowed good separation and reproducible quantification of amino acids by CE as OPA derivatives, and also permitted easy estimation of the rate constants for the derivatization of amino acids with OPA. Though its capabilities were not necessarily the best among the three possible techniques of in-capillary derivatization, they are high enough for practical analyses and kinetic studies.

References

[1] L.R. Tjaden, N.J. Reinhold, J. van der Greef, presented at 3rd symposium on Column Liquid Chromatography, Stockholm, Sweden, 25–30 June 1989.
[2] H.A. Fischman, J.B. Shear, L.A. Colon, R.N. Zare, US Pat. 5 318 680 (1994).
[3] S.D. Gilman, A.G. Ewing, Anal. Chem. 67 (1995) 58.
[4] A. Taga, S. Honda, J. Chromatogr. A 742 (1996) 243.
[5] A. Taga, M. Sugimura, S. Honda, J. Chromatogr. A 802 (1998) 243.
[6] B.J. Harmon, I. Leesong, F.E. Regnier, J. Chromatogr. A 726 (1996) 193.
[7] D.H. Patterson, B.J. Harmon, F.E. Regnier, J. Chromatogr. A 732 (1996) 119.
[8] S. Oguri, S. Watanabe, S. Abe, J. Chromatogr. 790 (1997) 177.
[9] M. Roth, Anal. Chem. 43 (1971) 880.