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Separation and enantiomer determination of *OPA*-derivatised amino acids by using capillary zone electrophoresis

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Summary. Amino acids react with OPA and chiral mercaptans to give diastereomeric isoindole derivatives. The resolution of these diastereomers was investigated by micellar electrokinetic chromatography (MECC) and free solution capillary electrophoresis. MECC with SDS as micellar phase allows to separate the amino acid derivatives and to resolve the diastereomers. The separation is influenced by the amount of detergent and the organic modifier added. Capillary zone electrophoresis offers a valuable alternative to the traditional methods for amino acid analysis and enantiomer determination.

Keywords: Amino acids – Capillary zone electrophresis – Micellar electrokinetic chromatography– OPA-derivatives – Enantiomer determination

Introduction

Amino acid analysis has become of great importance in the recent years for biochemical research and pharmaceutical industry. There has also been an increasing interest in stereoselective information which led to a demand for specific and sensitive methods to not only determine the individual amino acids but simultaneously to identify their stereomeric form. Gas chromatography [1] or high performance liquid chromatography [2–6] are commonly used for this purpose, but in the recent years also capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MECC) has found an increasing interest in analysing this type of compounds [7–11].

Generally, enantiomer determination can be accomplished by either using chiral stationary phases, chiral eluents or derivatisation with a chiral reagent prior to analysis. In the latter case diastereomers are formed which can often be separated by achiral separation techniques. One of the chiral derivatisation reagents is OPA together with N-acetyl-cysteine or Boc-Cysteine (OPA/NAC or OPA/BocC) which reacts with primary amino acids to diastereomeric isoindole derivatives as shown in Fig. 1. These diastereomeres could be successfully separated on conventional reversed phase HPLC [2, 3]. The type of deriva-

tisation is also applicable to other chiral mercaptans [4, 5]. In the present study, we explored the possibilities to separate the diastereomeric OPA derivatives by capillary zone electrophoresis and by MECC. Also the effect of positively charged additives and organic modifiers in the buffer were investigated.

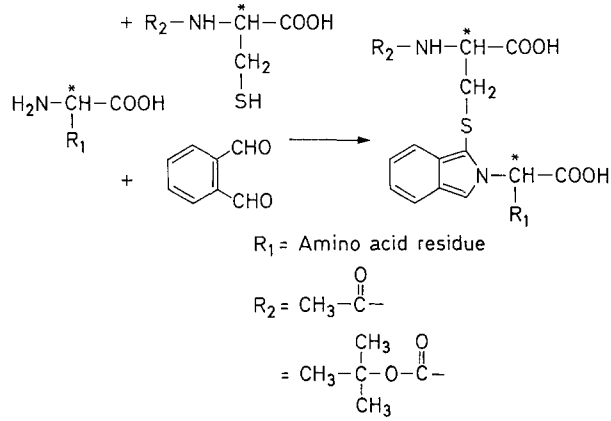

Fig. 1. Reaction of amino acids with OPA and a chiral mercaptan

Experimental

Apparatus

The separation was carried out on a Spectra Phoresis 1000 capillary zone electrophoresis instrument from Spectra-Physics Ltd. USA. The instrument was equipped with an on-column UV detector for multiwavelength detection. The capillaries were of uncoated fused-silica of 70 cm length and 75 μm I.D. purchased from Spectra-Physics. Data collection was performed using the PERKIN-ELMER integration system.

Chemicals

The amino acids were purchased from SIGMA (London, UK) or from SERVA (Heidelberg, FRG). Sodiumdodecyl sulfate (SDS) also was from SERVA. Tetrabutylammonium chloride (TBAC), tetrapropylammonium hydroxide (TPAH) and N-acetyl-L-cysteine (NAC) were from Fluka (Buchs, Switzerland), tetramethylammonium hydroxyde from Sigma (London, UK), o-Phthaldialdehyde (OPA) and boric acid from Merck (Darmstadt, FRG); N-tert-butyloxycarbonyl-L-Cysteine (Boc-L-Cysteine, BocC) from nova biochem (Laeufelfingen, Switzerland). Sodium borate buffer (pH 10) was prepared from boric acid by adjusting the pH with 1 N NaOH.

Derivatisation reagents

A 30-mg amount of OPA was dissolved in 1 ml of ethanol and diluted with 22 ml borate buffer (pH 10). To this solution 30 mg of the chiral mercaptan (NAC or BocC) were added. The reagents were kept in the refrigerator and replaced every other day.

Derivatisation procedure

100 μl of amino acid solution (approx. 3 μmol/ml of each amino acid) were mixed with 400 μl derivatisation reagent. After a reaction time of 10 min, the solution was injected in the CZE system.

Capillary cleaning and separation procedure

The capillary was washed with 0.1N NaOH and rinsed with water for two minutes after every run. Prior to the next injection the capillary was equilibrated with running buffer for two minutes. Sample injection was done by vacuum over 0.5 sec, the applied voltage was 20 kV if not stated otherwise. Capillary temperature was set to 30°C, detection occured with UV at 340 nm.

Results and discussion

OPA reacts rapidly with primary amino acids in the presence of a mercaptan to form thiosubstituted isoindoles [12]. These isoindoles are commonly measured by fluorescence detection, but they may also be detected by UV absorption. As example the UV spectrum of histidine after derivatisation with OPA/NAC is given in Fig. 2. The compound shows two absorption maxima, one at 230 nm the other one at 340 nm. Since the the OPA absorption interferes at 230 nm, the wavelength 340 nm was chosen for monitoring, even though the sensitivity at 340 nm is about three times less than at 230 nm.

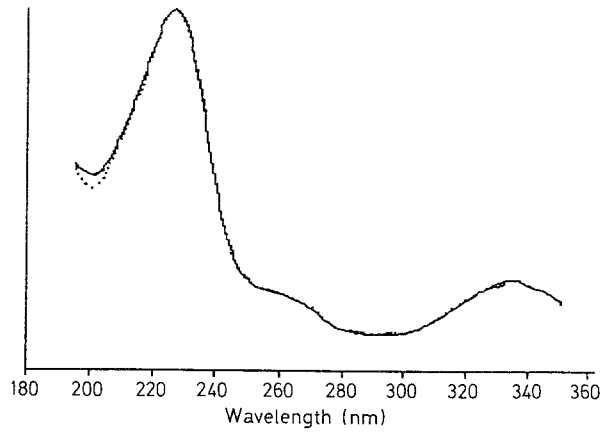

Fig. 2. UV spectrum of OPA/NAC-derivatised histidine

If amino acids are derivatised with OPA and a chiral mercaptan, the resulting products are diastereomers. The pairs of diastereomers bear identical charges and differ only in their stereochemistry. It is possible to separate the diastereomers by high performance liquid chromatography (HPLC) on conventional reversed phase columns [2–5]. In the present study we evaluated CZE as an alternative separation technique for this type of compounds.

By using free solution capillary electrophoresis a partial separation could be achieved only. It is possible to separate amino acids which differ in their charge, like Glu from Leu, Trp, Thr, Val, Ser as shown in Fig. 3. Amino acids bearing the same charge could not be sufficiently separated, neither a separation of the diasteromeric forms could be achieved. This is in agreement with the theory, as in free solution electrophoresis the difference in charge is the main factor for the separation.

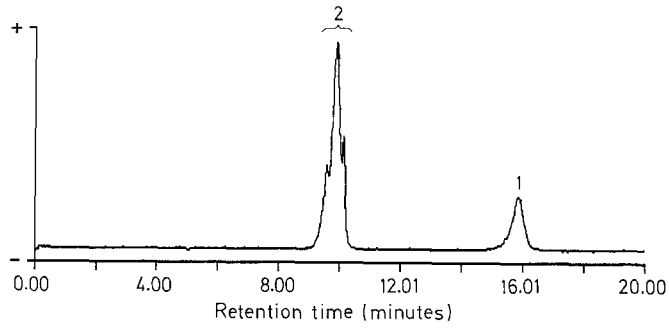

Fig. 3. Electropherogram of OPA/NAC derivatives of six amino acids *1* Glu, *2* Leu, Trp, Thr, Val, Ser. Buffer: 40 mM borate buffer pH 9.5, voltage 15 kV, current 122 μA, capillary 70 cm × 75 μm, temperature 30°C, detection 340 nm

Besides free solution capillary electrophoresis, also micellar electrokinetic chromatography (MECC) can be used for the amino acid separation. MECC is a technique initially introduced by Terabe et al [13] for the separation of neutral compounds. But the same principle is equally applicable to separate charged species. The technique involves the addition of an ionic surfactant to the running buffer in a concentration above the critical micelle concentration (CMC). The surfactant creates a micellar phase within the mobile buffer phase and the amino acids will distribute between the buffer phase and the micellar phase. Sodiumdodecyl sulfate (SDS) or bile acids are mainly used as micelle forming agent as reported in literature [11, 13, 14]. We have chosen SDS for our experiments and noticed a dramatic improvement in selectivity when using it. The presence of 0.15 M SDS in the running buffer allows to separate a number of amino acids and also resolves the diastereomeric forms. Furthermore, the peak capacity increased because the micelles migrate in the direction opposite to the electroosmotic flow.

A further improvement in selectivity was found by adding methanol in the running buffer. This is shown in Fig. 4 where electropherograms with methanol and without methanol are compared. Without adding methanol to the running buffer the derivatives of L-Leu and L-Val eluted in a single peak, whereas adding 5.5% methanol in the buffer led to a baseline separation of these two compounds and to a general improvement in selectivity. On the other hand the presence of methanol significantly increased retention time, probably due to its influence on the osmotic flow. Various methanol concentrations were evaluated and an amount of 5.5% methanol was found the optimum with respect to the separation of L-Leu/L-Val and the increase in retention time. A typical electropherogram of a separation of OPA/BocC-derivatised amino acids is shown in Fig. 5, and a list of the retention times is given in Table 1. For most of the derivatives a diastereomer separation could be reached and the L-forms always eluted before the corresponding D-forms. For the diastereomers of Asp, Asn, Ser and Thr no resolution could be achieved. These are the hydrophilic amino acids and it is assumed that the chiral centers of these compounds show insufficient interaction with the SDS micelles.

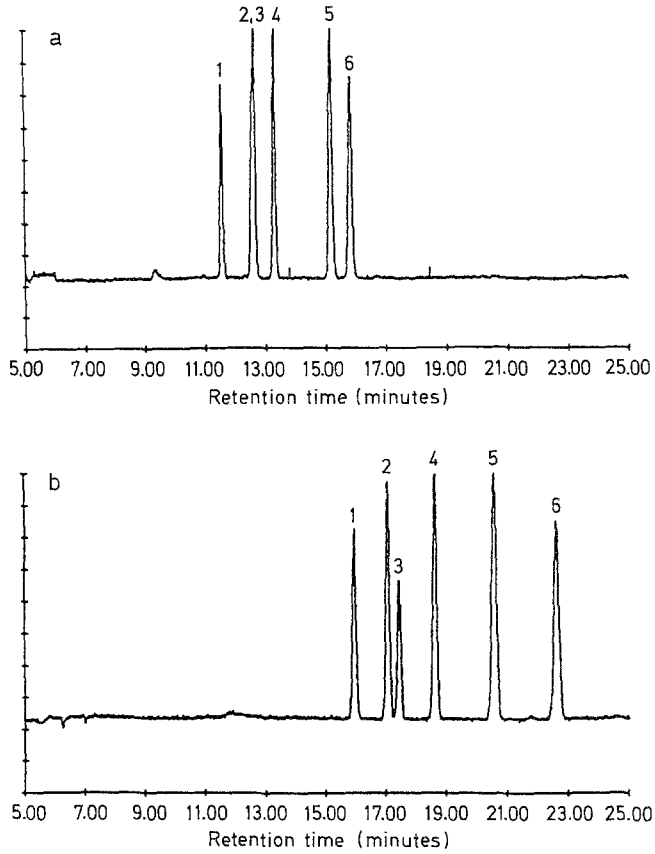

Fig. 4. Electropherogram of OPA/BocC derivatives of three DL amino acids without and with methanol in the running buffer. *1* L-Trp, *2* L-Leu, *3* L-Val, *4* D-Val, *5* D-Trp, *6* D-Leu, Buffer: 7.5 mM borate buffer pH 9.5 with 0.15 M SDS without methanol (**a**), and with 5.5% methanol (**b**), voltage 20kV, current 90 μA

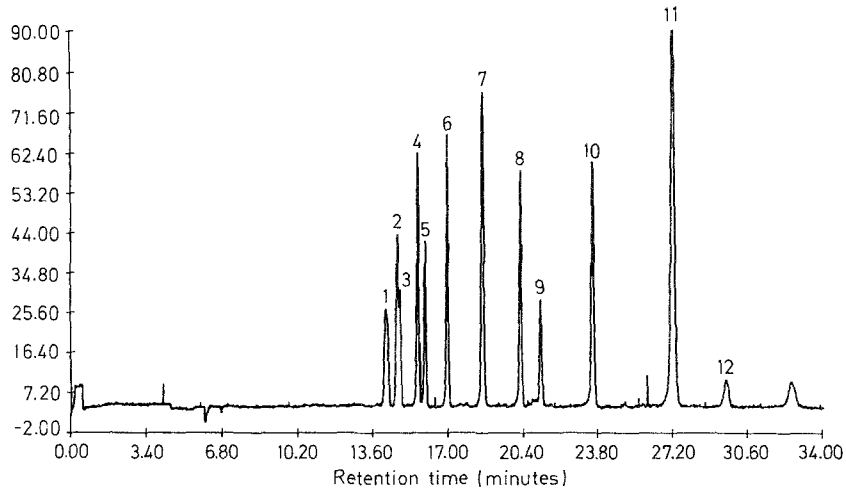

Fig. 5. Electropherogram of a mixture containing six amino acids derivatised with OPA/BocC. *1* L-Tyr, *2* D-Tyr, *3* L-Trp, *4* L-Leu, *5* L-Val, *6* D-Val, *7* D-Trp, *8* D-Leu, *9* L-Arg, *10* L-Lys, *11* -D-Lys, *12* D-Arg. Buffer: 7.5 mM borate buffer pH 9.6 with 0.15 M SDS and 5.5% methanol, voltage 20 kV, current 90 μA

Table 1. Retention time of OPA/BocC-derivatised amino acids

Amino acid	Retention time (min) L-form	D-form
Asn	13.25	(D, L coeluted)
Tyr	14.19	14.70
Gln	14.41	14.51
His	14.84	15.02
Trp	14.88	18.53
Asp	15.01	(D, L coeluted)
Thr	15.29	(D, L coeluted)
Leu	15.63	20.25
Ser	15.63	(D, L coeluted)
Val	15.96	16.96
Ala	16.04	16.49
Ile	16.08	18.78
Glu	16.17	16.86
Phe	16.35	18.40
Met	16.44	18.56
Arg	21.17	29.61
Lys	23.55	27.18

In addition to these experiments we also evaluated positively charged additives for their influence on the separation. Various tetraalkylammonium salts were chosen for this. Adding TMAH did not improve the resolution but when moving to more hydrophobic additives like TPAH or TBAC selectivity improved and a separation of a number of amino acid enantiomers could be achieved. TBAC was found hereby superior to TPAH because TBAC develops a better amino acid selectivity and a better diastereomer resolution. Fig. 6 shows a typical separation in the presence of TBAC.

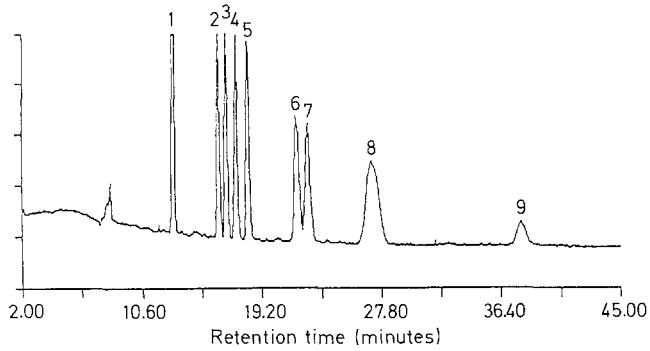

Fig. 6. Electropherogram of a diastereomer separation with TBAC in the running buffer. *1* DL-Trp, *2*, *3* DL-Leu, *4*, *5* DL-Thr, *6*, *7* DL-Val, *8* DL-Ser, *9* DL-Glu. Buffer: 30 mM borate buffer pH 9.5 with 1.5 mM TBAC, voltage 25 kV, current 107 μA

The separation effect of the positively charged additive is assumed to be related to a dynamic coating of the capillary wall. In normal fused silica

capillaries the wall is negatively charged, resulting from the dissociation of the silanol groups. A positively charged additive will now interact with the capillary wall to form a kind of dynamic coating on the wall. This decreases the electro-endosmotic mobility and simoultaneously leads in case of TPAH and TBAC to a hydrophobic coating which may undergo a hydrophobic interaction with the sample molecule. And it is assumed that such a hydrophobic interaction is responsible for the enantiomer resolution in presence of the positively charged additives.

Conclusion

The study shows that it is possible to resolve OPA-derivatised amino acid diastereomers by capillary zone electrophoresis. Essentially hereby is the presence of micelle forming agents or positively charged additives in the buffer. Best resolution is possible by adding SDS, which leads to the formation of micelles in the buffer and to a separation based on micellar electrokinetic chromatography. Positively charged additives also allow to resolve a number of amino acids. It is assumed that the positively charged additives form a hydrophobic layer on the capillary wall, such that the separation effect is based on the interaction of the sample molecule with the hydrophobic layer. Compared to HPLC, CZE is not able to resolve all amino acid enantiomers. On the other hand, some amino acids show a better resolution in MECC than in HPLC. Depending on the actual separation problem, the HPLC or the CZE/MECC technique may be advantageous.

References

1. Abdalla S, Bayer E, Frank H (1987) Chromatographia 23: 83
2. Buck RH, Krummen K (1984) J Chromatogr 315: 279
3. Buck RH, Krummen K (1987) J Chromatogr 387: 255
4. Brückner H, Wittner H, Godel H (1989) J Chromatogr 476: 73
5. Euerby MR, Patridge LZ, Gibbons WA (1989) J Chromatogr 483: 239
6. Einarsson S, Josefsson B, Möller P, Sanchez D (1987) Anal Chem 59: 1191
7. Gassmann E, Kuo JE, Zare RN (1985) Science 230: 813
8. Gozel P, Gassmann E, Michelson H, Zare RN (1987) Anal Chem 59: 44
9. Liu J, Cobb KA, Novotny M (1988) J Chromatogr 468: 55
10. Tran D An, Blanc T (1990) J Chromatogr 516: 241
11. Terabe S, Shibata M, Miyashita Y (1989) J Chromatogr 480: 403
12. García Alvarez Coque MC, Medina Hernández MJ, Villanueva Camañas RM, Mongay Fernández C (1989) Anal Biochem 178: 1
13. Terabe S, Ozaki H, Otsuka K, Ichikawa K, Tsuchiya A, Ando A (1984) Anal Chem 56: 111
14. Nishi H, Fukuyama T, Matsuo M, Terabe S (1990) J Chromatogr 515: 233

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