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Determination of chiral reagent purity by capillary electrophoresis

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Abstract

A method for the determination of the enantiomeric purity of the chiral reagent 1-(9-fluorenyl)ethyl chloroformate (FLEC) has been developed. The reagent is reacted with glycine, an achiral compound, and separated by capillary electrophoresis using β- or γ-cyclodextrin as chiral selectors. A general equation for the recalculation of the measured values with respect to the chiral reagent purity is presented. The suitability of this approach is practised on the peptide D-Arginine–Glycine to determine trace amounts of the enantiomeric contaminant, the L-form.

1. Introduction

The determination of enantiomeric purity is of increasing importance in the pharmaceutical industry where the concentration of an enantiomeric impurity often may not exceed 0.1%. Chiral impurity quantitation below 0.1% will most probably be enforced by regulatory agencies in most countries [1]. Regulations in this area are enforced because of the different biological activities of the enantiomers.

Enantiomeric vasopressin peptides sometimes show quite different biological activities. Essential differences in this respect have been observed in blood pressure activity of the natural hormone 8-Arg-vasopressin. The change of the amino acid residue in position 8 from the L- to the D-form resulted in a decrease in the blood pressure activity from 450 IU/mg to 4 IU/mg [2]. In liquid phase synthesis, the peptides are generated from the C-terminal (amide function) by fragment condensation. In order to control the biological activity during production of the active substance, it is important to determine any contamination of the corresponding enantiomer in the D-Arginine–Glycine (D-Arg–Gly) fragment at an early stage in the synthesis.

Enantiomeric determination of dipeptides in capillary electrophoresis (CE) has only been described by Tran et al. [3]. Their work concerns the separation of diastereomers after derivatisation with Marfey's reagent. The impurity of commercial Marfey's reagent has been reported to be approximately 0.25% of the opposite enantiomer [4]. When using chiral derivatising agents the enantiomeric purity of the reagent has to be exactly known, or the reagent has to be sufficiently pure in order to obtain an accurate determination of the enantiomeric ratio [5]. However, no published work on chromatograph-

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ic determination of chiral reagent impurity is known to the authors.

The drawback of reagent purity is eliminated in direct chiral separation. In a recently published paper, a comparison was made between direct and indirect chiral separation of amino acids by CE [6]. A large number of amino acids could be separated in both modes. The indirect method generally provided higher separation efficiencies than the direct method. The slow kinetics, generally connected with the direct chiral separation mechanisms, resulted in decreased chromatographic efficiency. Moreover, the screening procedure, which is necessary in order to find selectivity, was simplified by using the indirect approach.

The 1-(9-fluorenyl)ethyl chloroformate (FLEC) reagent has been briefly discussed as a versatile reagent in connection with micellar electrokinetic chromatography (MEKC) [7]. The advantages of the reagent have been discussed previous [6,8,9]. The commercially available FLEC reagent has a specified enantiomeric ratio of >99.5:0.5. High demands are made on a method for the exact determination of enantiomeric trace impurity. There are only a few papers dealing with the application of CE for the determination of enantiomeric trace impurity. Houben et al. [10] showed that such determinations are dependent on the detection sensitivity while the amount of sample to be introduced is limited. In addition, some different approaches in connection with pharmaceutical products have been investigated by Altria and co-workers [11–13]. Enantiomeric trace impurity determination of amino acids was reported by Ruyters and Van der Wal [14]. They developed a method for direct chiral separation of amino acids, derivatised with 4-fluoro-7-nitrobenz-2,1,3-oxadiazole, using β-cyclodextrin (β-CD) as chiral selector.

The aim of this work was to determine the enantiomeric purity of the chiral reagent FLEC in order to assure the enantiomeric quality of the hormone 8-D-Arg-vasopressin at trace levels. In this study emphasis is directed to find optimal CE separation conditions by statistical techniques. Then it is practicable to locate a specific position of a local optimum condition with full factorial design in combination with response surface modelling [15,16]. Experimental designs have previously been used for MEKC method developments [17,18].

2. Experimental

2.1. Chemicals

The (+)- and (−)-FLEC reagent was a gift from EKA Nobel AB (Bohus, Sweden). The Arg–Gly dipeptide was from Ferring Pharmaceuticals (Malmö, Sweden). Boric acid, phosphoric acid and 2-propanol were from E. Merck (Darmstadt, Germany). Sodium dodecyl sulphate (SDS) from Fluka Chemie AG (Buchs, Switzerland). Glycine, β-, and γ-cyclodextrin (β-, and γ-CD) were from Sigma (St. Louis, MO, USA). All buffer solutions were made with water from an Elgastat UHQII (Elga, High Wycombe, UK).

2.2. Apparatus

The separations were carried out using a Prince autosampler (Lauerlabs, Emmen, Netherlands). An ISCO CV^4 UV detector (ISCO, Lincoln, NE, USA) was used at 256 nm for the detection. The separation capillaries (25 and 50 μm I.D.) were from Polymicro Technologies (Phoenix, AZ, USA). Data collection was made with ELDS 900 (Chromatography Data System, Kungshög, Sweden).

2.3. Derivatisation procedure

A solution of 10 m*M* Arg–Gly in 200 m*M* borate buffer (pH 9.2) was prepared. From this solution 400 μl was mixed with 400 μl of 30 m*M* FLEC reagent solution in acetone. After 10 min the reaction mixture was extracted with 0.5 ml pentane to terminate the reaction by removing excess reagent. The aqueous phase was diluted 10× with water and thereby ready for injection. The derivatisation of glycine was performed in the same way.

2.4. Separation conditions

The capillary was rinsed with five column volumes of 0.1 *M* NaOH and water, then equilibrated with ten column volumes of buffer prior to each run. For other separation conditions see figure legends. Buffer concentration, SDS concentration, CD concentration and pH are given as they were before the addition of organic modifiers. All injections were made by pressure.

2.5. Optimisation

Calculations and figures concerning optimisation were performed using the CODEX program (AP Scientific Service, Sollentuna, Sweden). The starting point for the optimisation of dipeptide MEKC separation was identical to separation conditions found for FLEC derivatised D- and L-arginine [6]. The test experiments were made by varying pH, buffer composition, SDS concentration, type of organic modifier and organic modifier concentration. When selectivity was found, a central composite design with three variables in two levels was used in order to optimise the separation system.

For the direct chiral separations, type of CD, CD concentration, pH, buffer composition, type and amount of organic modifier were varied in the scouting experiments. Optimisation was performed in the same way as for MEKC, except that only two variables were used.

The fit of the models to experimental data was evaluated by making five runs in the centre point of the models. The experimental noise was thereby determined. In addition to the response surfaces, the 95% confidence interval is represented as the interval over the regression coefficient. If the interval exceeds the regression coefficient, the variable is not significant to the model.

The functional relation between the experimental variables and the obtained results is approximated to fit a Taylor expansion

$$y = \beta_0 + \sum \beta_i x_i + \sum \sum \beta_{ij} x_i x_j + \beta_{ii} x_i^2 + e$$

where the coefficients β are the parameters of the model and e is the overall error term [16]. The estimation of the parameters is done using multiple linear regression and a polynomial model is fitted to the experimental results. The linear coefficient for the experimental variables, β_i, describes their quantitative influence of the model. The cross-product, β_{ij} will measure the interaction effect between the variables, and the square term $\beta_{ii}x_i^2$ will describe the non-linear effect of the response.

3. Results and discussion

3.1. Determination of enantiomeric purity of (+)- and (−)-FLEC by reaction with glycine

When diastereomers are formed, reagent enantiomeric impurity will contribute to errors in the analytical results. This is due to the fact that the impurity will react and form enantiomeric pairs with the main products. The enantiomers will coelute and affect the impurity peak more than they do the main peak [5]. However, if the purity of the chiral reagent is known, the enantiomeric purity of the compound of interest can be calculated by:

$$y = \frac{1}{2}\left[\frac{M_A - I_A}{(2x-1)(M_A + I_A)} + 1\right] \quad (1)$$

where y is defined as the molar fraction of one enantiomer $[y = R/(R+S)]$ of the compound to be investigated, and x is defined in the same way as the molar fraction of one form of the reagent $[x = R/(R+S)]$. M_A and I_A are the peak areas (i.e. corrected peak areas) of the main and impurity peak, respectively.

In the case where $x = 1$ (100% enantiomeric purity of the reagent) Eq. 1 is reduced to:

$$y = \frac{1}{2}\left[\frac{M_A - I_A}{M_A + I_A} + 1\right] = \frac{M_A}{M_A + I_A} \quad (2)$$

In order to develop an accurate method for the enantiomeric trace-impurity determination utilising the benefits of the diastereomeric approach, the (+)- and (−)-FLEC reagent was reacted with glycine, an amino acid without a

chiral centre, thus producing (+)- and (−)-FLEC-glycine (FLEC-Gly) enantiomers. No differences in reaction speed can occur since the products possess the same physicochemical properties.

In the separation of the reagent it was found that the selectivity changed with β- and γ-CD. However, the presence of 2-propanol in the buffers was necessary for selectivity. The elution order of the FLEC-Gly enantiomers changed completely with the different cyclodextrins.

The separations were optimised with a factorial design including the 2-propanol and β- or γ-CD concentration, respectively. The pH was not included since the test runs did not show a significant effect of the pH in the range 5.5–7.0. Fig. 1 shows the response surfaces for β-CD (A) and γ-CD (B) as a function of the 2-propanol concentration with the resolution as the response. The optimisation for the β-CD concentration in Fig. 1A showed that the resolution increased with increasing β-CD concentration and that an optimum 2-propanol percentage existed. Since a concentration of β-CD above 15 m*M* resulted in increased noise, 14 m*M* β-CD and 18% 2-propanol were chosen for running conditions. A separation under these conditions is presented in Fig. 2A.

The elution order of (+)- and (−)-FLEC-Gly was altered when β-CD was changed to γ-CD. The best resolution was found with 12% 2-propanol and 10 m*M* γ-CD (Fig. 2B). The change in the elution order of (+)- and (−)-FLEC is important in order to determine the purity of both (+)- and (−)-forms, since it allows for the small impurity peak to be eluted before the main peak. With γ-CD as the chiral selector, the enantiomeric purity of 1.0 m*M* (+)-FLEC-Gly was investigated. Under these conditions no impurity peak was observed. However, after standard addition of 0.01% (−)-FLEC-Gly, a peak was observed, (Fig. 3). Since the purpose was to show the presence of (−)-FLEC-Gly a signal-to-noise ratio of 2/1 was used.

With β-CD as chiral selector, the (+)-FLEC-Gly was eluted first. The purity of 1.0 m*M* (−)-FLEC-Gly was investigated. Under these conditions no impurity peak was observed. However, after standard addition of 0.1% (+)-FLEC-Gly, a peak was observed (Fig. 4). Determination below 0.1% was not successful. This is mainly caused by the lower resolution obtained with β-CD than with γ-CD. The lower resolution led to a partial overlap of the peaks.

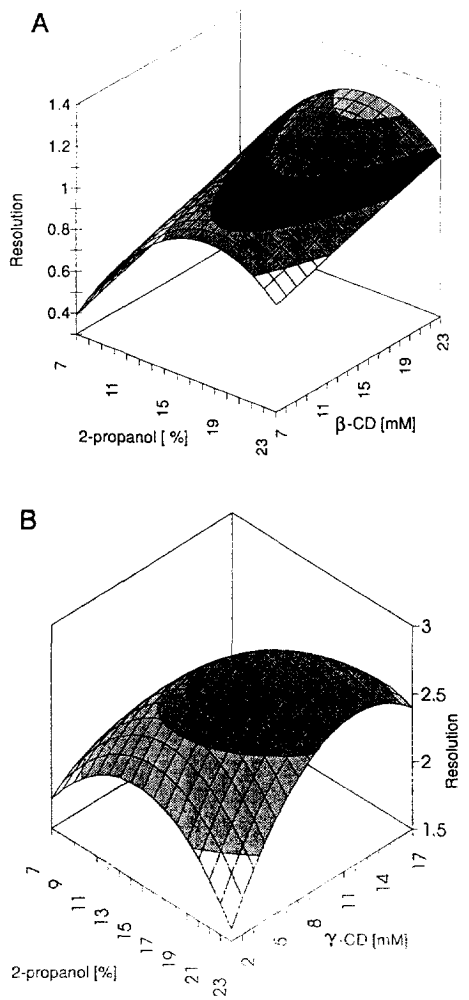

Fig. 1. Optimisation of separation of (±)-FLEC-Gly. Separation column: 50 μm I.D. × 65 cm (45 cm to detector); Voltage: 25 kV; Buffer: 50 m*M* phosphate, pH 6.0; 2-propanol and CD concentrations are varied. (A) β-Cyclodextrin used as chiral selector, (B) γ-cyclodextrin used as chiral selector.

The relative standard deviations of the repeatability of the determination of two (−)-FLEC-Gly spiked samples, were 14.6 and 5.8 for 0.1% and 0.5%, respectively ($n = 6$).

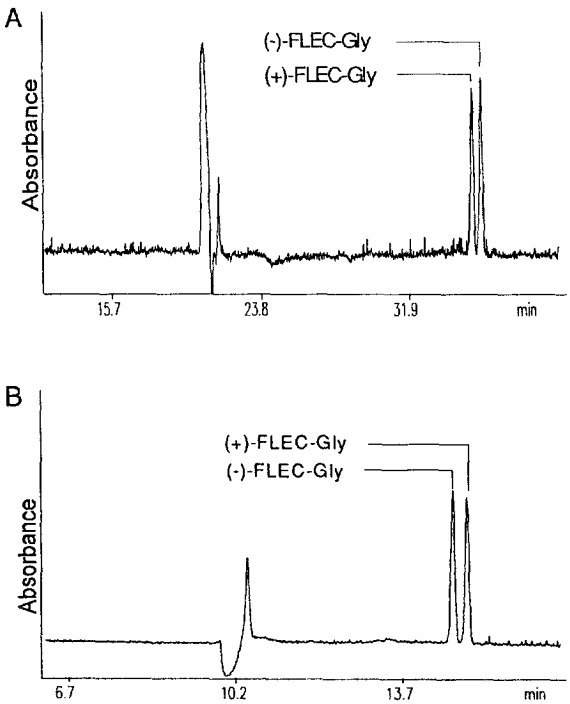

Fig. 2. Separation of (±)-FLEC-Gly. Column: 50 μm I.D. × 65 cm (45 cm to detector). (A) Buffer: 100 m*M* acetate, pH 6.0, 14 m*M* β-CD, 18% 2-propanol; 20 kV, 18 μA. (B) Buffer: 50 m*M* phosphate, pH 6.0, 10 m*M* γ-CD, 12% 2-propanol; 20 kV, 16 μA.

3.2. Diastereomeric separation of dipeptide

In order to obtain the optimum separation conditions, an experiment was performed by

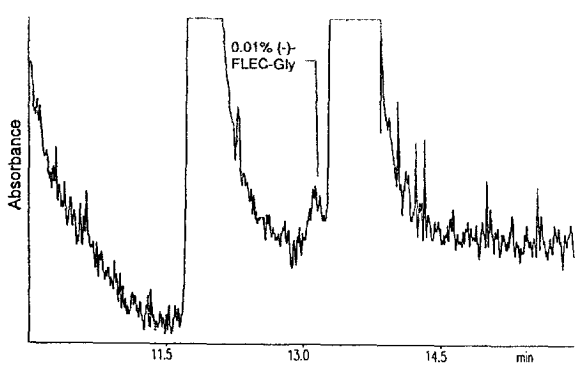

Fig. 3. Determination of 0.01% (−)-FLEC-Gly added to a (+)-FLEC-Gly sample. Buffer: 50 m*M* phosphate, pH 6.0, 10 m*M* γ-CD, 12% 2-propanol;. 20 kV, 16 μA. Column: 50 μm I.D. × 65 cm (45 cm to detector).

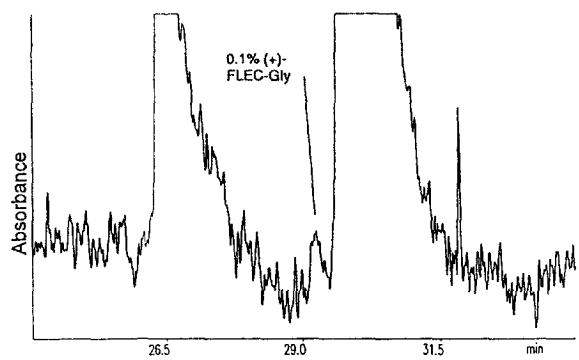

Fig. 4. Determination of 0.1% (+)-FLEC-Gly added to a (−)-FLEC-Gly sample. Buffer: 50 m*M* phosphate, pH 6.0, 14 m*M* β-CD, 18% 2-propanol; Separation column: 25 μm I.D. × 65 cm (45 cm to detector); 30 kV; 6 μA.

factorial design. According to the test runs the most important factors were the SDS, buffer and 2-propanol concentrations. These three factors were therefore chosen as the variables in the optimisation experiment.

Three different response surfaces from this experimental design are presented in Fig. 5. The calculated responses were: selectivity (A, calculated without micellar elution time, see Ref. [6]), efficiency (B, theoretical plate number), and resolution (C, calculated according to conventional methods). From the response surfaces the effects of the different buffer parameters are determined. The main effect of 2-propanol was found for the selectivity response (Fig. 5A). Although a high SDS concentration gave an increased selectivity, the main effect was that low SDS concentrations resulted in higher selectivity, (Fig. 5A). High efficiency was obtained with high concentration of SDS (Fig. 5B). The 2-propanol square term showed that an optimum existed for efficiency and a minimum for selectivity. The resolution response surface shown in Fig. 5C was mainly dependent on the 2-propanol and buffer concentrations. The separation of D- and L-Arg–Gly-FLEC under optimum conditions is shown in Fig. 6.

Determination of the chiral dipeptide impurity was performed from the chromatogram in Fig. 7. The amount of L-form in the D-Arg–Gly sample was found to be 0.013%, with a standard devia-

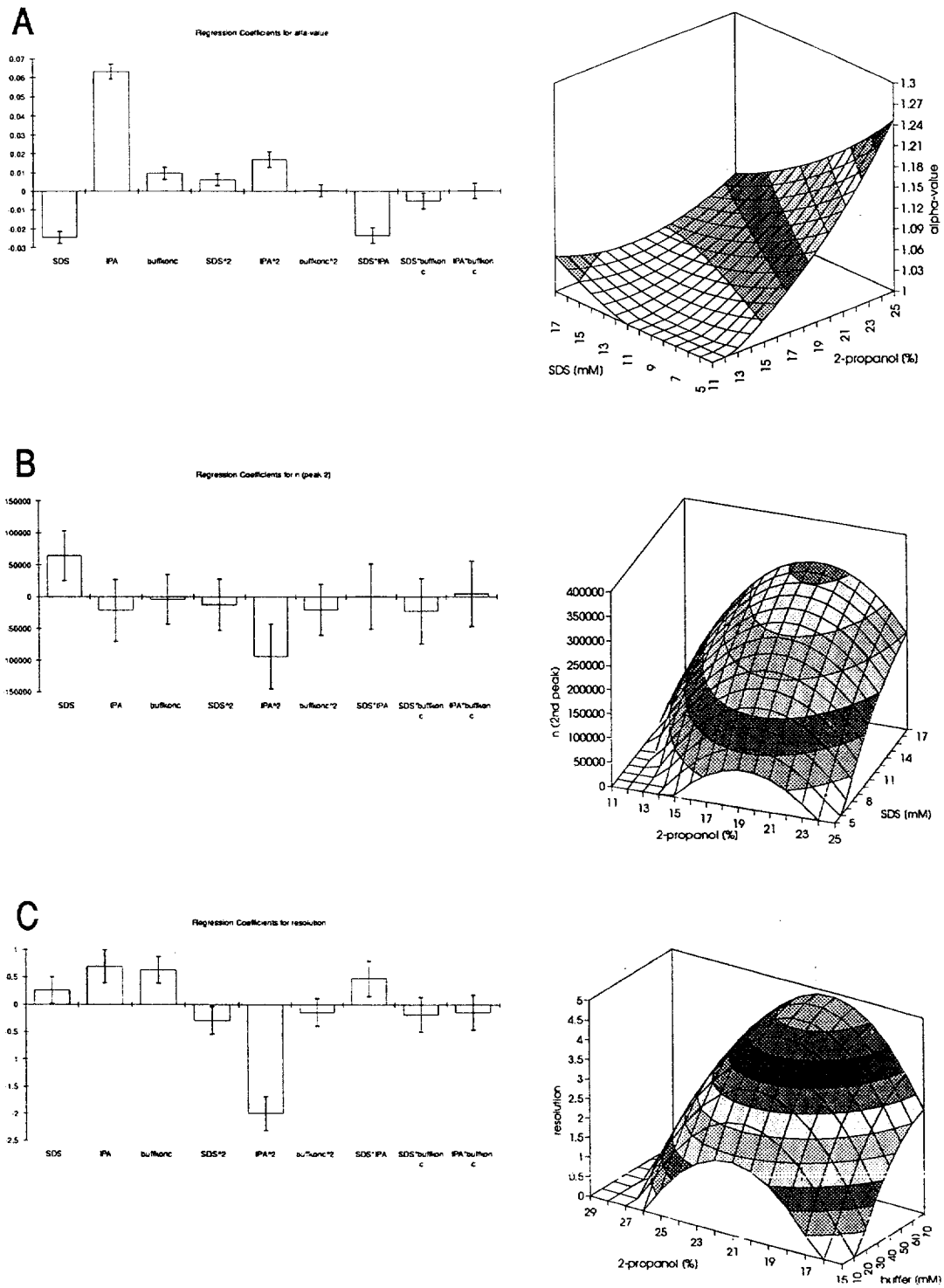

Fig. 5. Optimisation of FLEC-D,L-Arg–Gly separation. Separation column: 25 μm I.D. × 56 cm (40 cm to detector); pH 9.2; SDS, 2-propanol and buffer concentrations are varied.

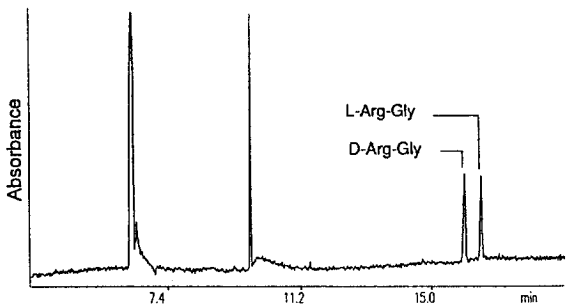

Fig. 6. MEKC separation of (−)-FLEC-D,L-Arg–Gly. Buffer: 20 m*M* borate–15 m*M* phosphate, pH 9.20, 10 m*M* SDS, 18% 2-propanol; Column: 25 μm I.D. × 56 cm (40 cm to detector); Voltage: 30 kV, 7.8 μA.

tion of 0.002%. The determination was carried out by the standard addition method using 4 points. The percentages added were: 0.024, 0.047 and 0.094, the regression coefficient was $r^2 = 0.9966$.

If the (+)-FLEC would contain an impurity of 0.01% (−)-FLEC, the impurity of L-Arg–Gly in the D-Arg–Gly would be 0.003% according to Eq. 1. However, the amount of (−)-FLEC could only be determined to be less than 0.01%; thus, in this case it is better to use Eq. 2 since the value for the dipeptide impurity will otherwise be too low.

The detection limit for FLEC-Gly and FLEC-Arg–Gly was 0.1 μ*M*. This is ten times lower than the results reported earlier for the analogues non-chiral derivatisation reagent FMOC

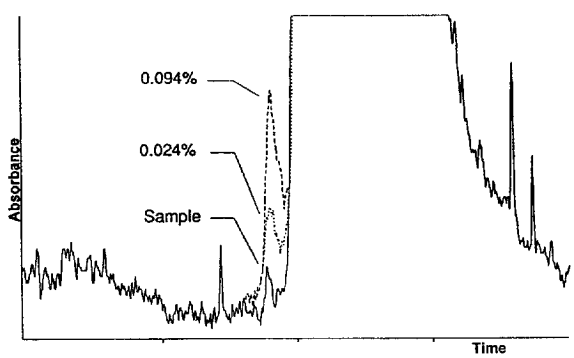

Fig. 7. Determination of L-Arg–Gly in a D-Arg–Gly sample by standard addition. Buffer: 20 m*M* borate–15 m*M* phosphate, pH 9.20, 10 m*M* SDS, 18% 2-propanol. Separation column: 50 μm I.D. × 65 cm (45 to detector); Voltage: 30 kV.

[19]. The sample plug injected was calculated to be 2.3 cm, which is one order of magnitude longer than what is generally recommended [20]. Stacking conditions were obtained without any desalting or pH adjustment of the sample prior to the injection. The buffer concentration in the sample was higher than in previously reported work [19]. In this work the higher buffer concentration remained from the derivatisation process.

4. Conclusions

The enantiomeric purity of chiral reagents at trace levels can be established by efficient separation systems. Capillary electrophoresis with chiral selectors such as cyclodextrins has been shown useful to separate (+)- and (−)-FLEC after reaction with achiral glycine.

By using the stacking principle a large amount of sample can be applied in the capillary; thus the (±)-enantiomers can be determined in a ratio of 1:10 000. Enantiomeric trace analyses are facilitated by a combination of efficient separation of the diastereomers and high detection sensitivity, which can be accomplished by incorporating a strong chromophore in the derivatising reagents. The FLEC reagent is available in both enantiomeric forms and thus the elution order of the derivatisation products can be arranged in a way that the small peak elutes in front of the large peak.

The indirect chiral separation with (+)- or (−)-FLEC reagents is demonstrated to be favourable to verify the enantiomeric purity of peptides at trace levels when the reagent enantiomeric purity is assured.

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References

[1] M. Novotny, H. Stefansson and S. Soini, Anal. Chem., 66 (1994) 641A.
[2] M. Zaoral, J. Kolc and F. Sorm, Collection Czech Chem. Commun., 31 (1966) 382.
[3] A.D. Tran, T. Blanc and E.J. Leopold, J. Chromatogr., 516 (1990) 241.
[4] L. Hansson and R. Isaksson, J. Chromatogr. A, 653 (1993) 9.
[5] S.G. Allenmark, Chromatographic Enantioseparation: methods and applications, Ellis Horwood Limited, Chichester, 1988.
[6] H. Wan, P.E. Andersson, A. Engström and L.G. Blomberg, J. Chromatogr. A, 704 (1995) 179.
[7] R. Weinberger, Practical Capillary Electrophoresis, Academic Press, San Diego, CA, 1993, p. 184.
[8] S. Einarsson, B. Josefsson, P. Möller and D. Sanchez, Anal. Chem., 59 (1987) 1191.
[9] C.W. Demarest, E.A. Monnot-Chase, J. Jiu and R. Weinberger, in P.D. Grossman and J. Colburn (Editors), Capillary Electrophoresis: Theory and Practice, Academic Press, San Diego, CA, 1992, Ch. 11, p. 336.
[10] R.J.H. Houben, H. Gielen and S.J. van der Wal, J. Chromatogr., 634 (1993) 317.
[11] K.D. Altria, Chromatographia, 35 (1993) 177.
[12] K.D. Altria, Chromatographia, 35 (1993) 493.
[13] M.M. Rogan, K.D. Altria and D.M. Goodall, Chromatographia, 38 (1994) 723.
[14] H. Ruyters and S.J. van der Wal, J. Liq. Chromatogr., 17 (1994) 1883.
[15] S.F.Y. Li, Capillary Electrophoresis, J. Chromatogr. Library Ser., Vol. 52, Elsevier, Amsterdam, 1992.
[16] R. Carlson, Design and Optimisation in Organic Synthesis, Elsevier, Amsterdam, 1992.
[17] J. Vindevogel and P. Sandra, Anal. Chem., 63 (1991) 1530.
[18] J.H. Jumppanen, S.K. Wiedmer, H. Siren, M.L. Riekkola and H. Haario, Electrophoresis, 15 (1994) 1267.
[19] A. Engström, P.E. Andersson, W.D. Pfeffer and B. Josefsson, Anal. Chem., (1995) in press.
[20] S.L. Delinger and J.M. Davis, Anal. Chem., 64 (1992) 1947.