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Determination of D-alanine and D-glutamic acid in biological samples by coupled-column chromatography using β-cyclodextrin as mobile phase additive

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ABSTRACT

A high-performance liquid chromatographic assay for monitoring traces of D-alanine and D-glutamic acid in peptide and protein hydrolysates is presented, which uses a two-column set-up with column switching. The main advantage of the proposed method is the higher reliability of the results, compared with the analysis by derivatization and single-column chromatography of the diastereomers. A non-chiral alkylsilica reversed-phase column is combined with a second column, in which β-cyclodextrin is used as a chiral mobile phase additive. The amino acids of the hydrolysate are dansylated, and the amino acid of interest is separated from the others on the first column and transferred to the second column where chiral resolution is performed. The transfer volume of *ca.* 200 μl is small enough not to cause any peak distortion or dilation in the second column.

INTRODUCTION

The determination of the content of D-amino acids in peptides or proteins has attracted broad interest in many fields. Among others, there are interesting new questions arising from genetics and molecular biology. D-Amino acids are known to occur in the peptidoglycan of cell walls of bacteria, where especially alanine (Ala) and glutamic acid (Glu) have been found in both L and D configurations [1]. Intensive investigations are now directed to the question of whether and to what extent D-amino acids exist in the cell wall proteins and peptides of eucaryotic cells.

Several different approaches have been proposed and successfully used for the determination of D-amino acids. They include gas chromatographic analysis after derivatization [2], high-performance liquid chromatographic (HPLC) analysis of the free acids by metal–chelate ligand-exchange [3], HPLC of amino acid derivatives employing various chiral recognition mechanisms [4–10] or HPLC after the formation of diastereomers by chiral derivatization agents [11,12].

The determination of compounds at low concentrations in the presence of other components or impurities by single-column chromatography

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always suffers because peak overlap cannot be completely excluded. This is due to the limited peak capacity of one column. Such uncertainties can be effectively reduced when using coupled-column chromatography (CCC) with column switching [13]. Such a coupled-column HPLC system is proposed here for the determination of D-Ala and D-Glu in protein hydrolysates.

Tapuhi *et al.* [14] have presented a CCC method for determining amino acids after derivatization with dansyl chloride, combining a non-chiral reversed-phase (RP) column with a subsequent chiral ligand-exchange system, in which the chiral selector is employed as a mobile phase additive. In this paper we propose a similar strategy, which combines the non-chiral RP column with a chiral system, where the chiral selector additive is β-cyclodextrin. The amino acids are investigated as the dansyl derivatives.

EXPERIMENTAL

Instrumentation

Chromatographic experiments were carried out with two HPLC pumps [(i) Model L-6200 intelligent pump (Merck-Hitachi, Hitachi, Tokyo, Japan) and (ii) Model 116 solvent module, System Gold (Beckman, San Ramon, CA, USA)], a syringe-valve injector (Model 7161, Rheodyne, Cotati, CA, USA) equipped with a 20-μl loop, two switching valves (Model 7030, Rheodyne) and a fluorescence detector (Model F-1000, Merck-Hitachi) connected to an integrator (Model D-2000 Chromato-integrator, Merck-Hitachi).

Columns and mobile phases

Two prepacked stainless-steel columns (250 mm × 4.0 mm I.D.) filled with chemically bonded octadecylsilica (ODS) obtained from E. Merck (Darmstadt, Germany) were used: column I, LiChrosorb RP-18 (7 μm particle diameter); column II, LiChrospher 100 RP-18 (5 μm particle diameter).

The mobile phase for column I was aqueous buffer (25 m*M* ammonium acetate, 0.1% triethylamine, pH 5.5)–ethanol (79:21, v/v) for monitoring dansyl-Glu and (70:30) for dansyl-Ala.

The mobile phase for column II was aqueous buffer (25 m*M* ammonium acetate, pH 5.5)–ethanol (80:20, v/v), containing 35 m*M* β-cyclodextrin and 1 *M* urea.

Reagents and samples

Water was distilled twice from a quartz apparatus and additionally purified by an Elgastat UHQ apparatus (Elga, High Wycombe, UK). Ethanol was obtained from E. Merck in LiChrosolv quality. Alcohol–water mixtures were partially premixed and degassed in an ultrasonic bath. Analytical-grade triethylamine, ammonium acetate and urea were used. β-Cyclodextrin was purchased from E. Merck, dansyl chloride from Sigma (St. Louis, MO, USA) and the (+)-1-(9-fluorenyl)ethylchloroformate [(+)FLEC] reagent was obtained from Fluka (Buchs, Switzerland). All eluent mixtures were filtered before use through a Nylon-66 membrane filter with 0.45-μm pore diameter (Supelco, Bellefonte, PA, USA).

Chromatographic procedure

A schematic presentation of the instrumental set-up is given in Fig. 1. A general chromatographic elution and column-switching protocol for this set-up is described in Table I[a]. The injection volume was 20 μl. Fluoresence detection was carried out with an excitation wavelength of 340 nm and emission at 480 nm. The temperature was ambient for both columns, and the flow-rate was 0.5 ml/min.

Sample pretreatment

Fungal spore walls were hydrolysed in 6 *M* HCl at 110°C for 12 h. HCl was removed under reduced pressure (*ca.* 0.5 Torr), and hydrophilic amino acids were prepurified by passing the hydrolysate through an RP column (DeltaPak C_{18}, Waters) using 0.1% trifluoroacetic acid as solvent. The detection wavelength was 214 nm.

[a] In principle, the same separations can be obtained by using a single pump and a capillary by-pass according to the set-up described previously [15]. In this case about twice the time is required for one analysis cycle.

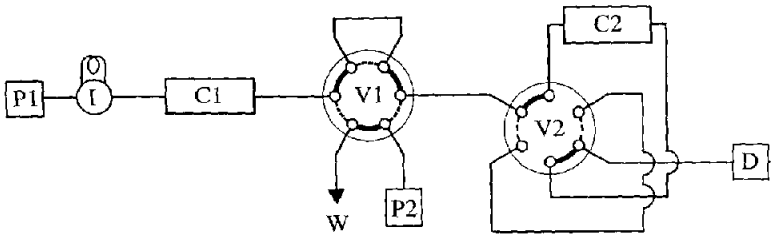

Fig. 1. Schematic diagram of the CCC system. Valves: rotor position A, bold lines; rotor position B, broken lines. Symbols: P1 and P2, pumps; C1 and C2, columns; V1 and V2, valves; I, injector; D, detector; W, waste.

TABLE I

GENERAL ELUTION AND COLUMN-SWITCHING PROTOCOL

Time interval[a]	Rotor position of valves[b]		Columns in operation[c]	Eluent[d]	Comment[e]
	Valve 1	Valve 2			
$0 - t_1$	A	B	1	1	A
$t_1 - t_2$	A	A	1 + 2	1	B
$t_2 - t_3$	A	B	1	1	C
$t_3 - t_4$	B	A	1	1 (or gradient)	D, E
			2	2	D, F

[a] t_1 = beginning of analyte transfer; t_2 = end of analyte transfer; t_3 = eluent stream from column 1 is switched off from the detector: end of the chromatogram of first column, start of a gradient program on pump 1 for washing and reconditioning column 1; the eluent stream from pump 2 is switched to operate column 2; t_4 = end of the chromatogram of column 2.

[b] Rotor positions, A and B, of the switching valves refer to Fig. 1.

[c] Column 1, RP column; column 2, ODS column operated with a chiral mobile phase (eluent 2).

[d] Eluent 1 (for column 1), aqueous buffer (25 m*M* ammonium acetate, 0.1% triethylamine, pH 5.5) with adjusted ethanol content; eluent 2 (for column 2), aqueous buffer (25 m*M* ammonium acetate, pH 5.5, 35 m*M* β-cyclodextrin, 1 *M* urea), 30% (v/v) ethanol.

[e] A = separation of the Dns-amino acids; B = transfer of a particular racemic amino acid; C = continuation of elution from column 1, a few minutes before t_3: start of pump 2 (this time interval can be kept very short); D = parallel working of pump 1 for column 1 and pump 2 for column 2; E = washing and reconditioning of column 1 by pump 1 using an appropriate gradient, *e.g.* step gradient with 70% of ethanol, before reconditioning with eluent 1; F = chiral separation employing eluent 2 delivered by pump 2.

Unretained material was collected, dried under reduced pressure and used for further analysis.

About 20 nmol of the amino acids, crudely prepurified in the described way, were dissolved in *ca.* 400 μl of a borate buffer solution (0.1 *M*, pH 9). After the addition of 300 μl of a solution of dansyl chloride in acetone (10 mg/ml) the solution was allowed to stand for 2 h in the dark. Thereafter it was dried under a nitrogen stream and redissolved in acetone–1 *M* HCl (19:1, v/v). The liquid phase was separated from the insoluble salts by centrifugation and dried again under nitrogen. For the complete elimination of the salts the extraction step can be repeated. The dry sample containing the dansylated amino acids was stored at −20°C. It was dissolved in the eluent mixture before injection.

RESULTS

Typical chromatograms obtained with the CCC system described are shown for the determination of the enantiomers of Ala in Fig. 2 and the enantiomers of Glu in Fig. 3. The chromato-

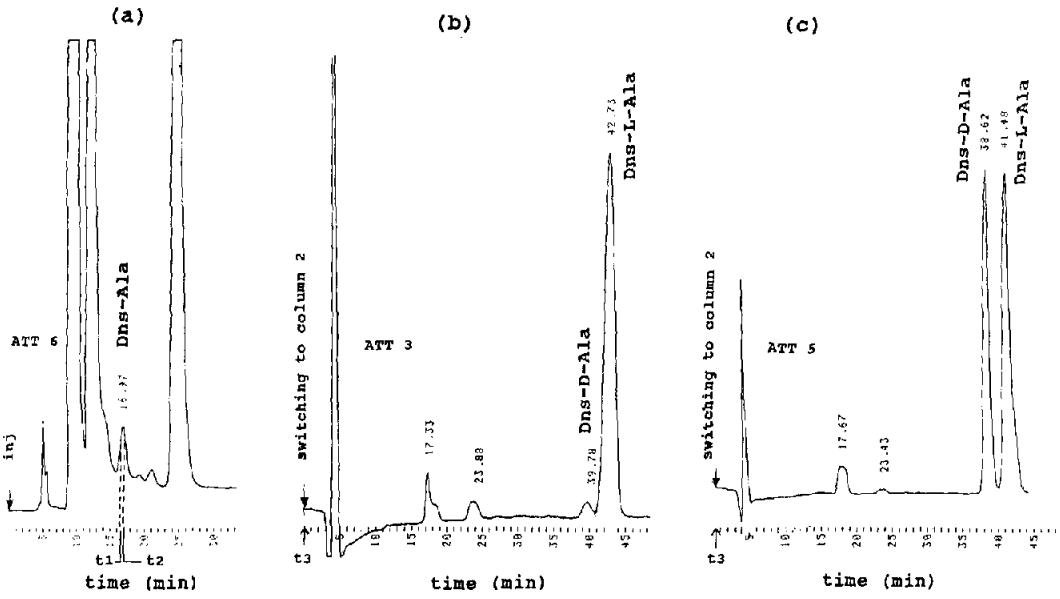

Fig. 2. Determination of Dns-D-Ala in a protein hydrolysate. (a) Chromatogram obtained from the first column: separation of the Dns-amino acids in the hydrolysate. The arrows indicate the switching times, t_1 and t_2, *i.e.* start and end of the transfer. (b) Chromatogram obtained from the second column: enantiomeric separation of Dns-Ala. (c) Chromatogram as in (b), except that the hydrolysate was spiked with D/L-Ala prior to dansylation. Chromatographic conditions as in Experimental. Times of switching events: t_1, 16.60 min, t_2, 17.00 min (for the definition of t_1, t_2 and t_3 see Table I).

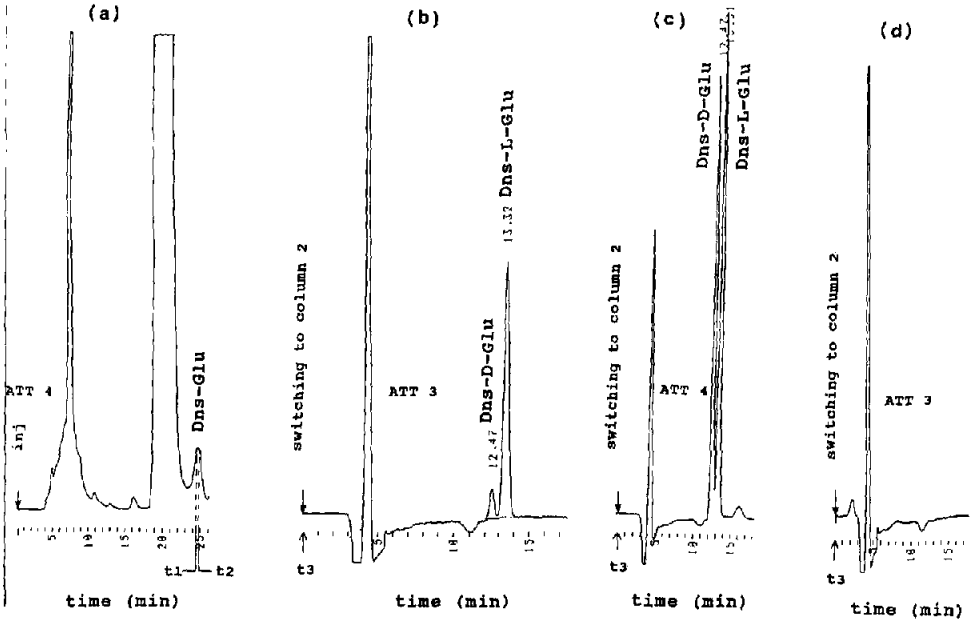

Fig. 3. Determination of Dns-D-Glu in a protein hydrolysate. (a) Chromatogram obtained from the first column: separation of the Dns-amino acids in the hydrolysate. The arrows indicate the switching times, t_1 and t_2, *i.e.* start and end of the transfer. (b) Chromatogram obtained from the second column: enantiomeric separation of Dns-Glu. (c) Chromatogram as in (b), except that the hydrolysate was spiked with D/L-Glu prior to dansylation. (d) Chromatogram as in (b) after injection of a blank sample. Chromatographic conditions as in Experimental. Times of switching events: t_1, 24.50 min; t_2, 24.80 min.

grams of the hydrolysates on column 2 show very few signals other than the analyte peaks of interest. The D-enantiomers are eluted first in both cases.

The volumes transferred were *ca.* 200 μl. Significantly higher volumes led to additional peak broadening and loss in resolution on the second column [16]. This limit allows the transfer of a part of the peaks only, which reduces the sensitivity of the determination. Narrow cuts, however, diminish the probability of overlapping peaks being co-transferred and this increases the reliability of the conclusions. This aspect is one of the main purposes of introducing a CCC method.

The percentage of D-Ala in Fig. 2 is 3.3%, and that of D-Glu in Fig. 3 is 9.2%[a]. One can thus expect that this method allows a fairly accurate determination of D-Ala down to *ca.* 1%, and of D-Glu down to 2%. The higher limit for D-Glu is due to the slight peak overlap under the chosen conditions.

When chiral mobile phase additives are used for the separation of enantiomers, the sensitivities of the detection of the two diastereomeric analyte–additive associates (complexes) are not generally equal. This also holds true for the complexes of β-cyclodextrin with dansylated amino acids. Under the chosen conditions D-Ala was detected more sensitively than L-Ala (by a factor of 1.28) and D-Glu more sensitively than L-Glu (by a factor of 1.21). These factors were determined from chromatograms of the racemic analytes.

DISCUSSION

The main scope of the proposed method is the accurate determination of the D-enantiomers of several amino acids, particularly Ala and Glu, in hydrolysates of proteins or peptides. The main features of the described method are: (i) it is easy and reasonably fast; the most time-consuming step is the dansylation procedure; (ii) dansyl derivatives allow the use of fluorescence detectors, which are more sensitive than UV detectors; (iii) the two-column technique significantly diminishes the probability of peak overlap by impurities or other components and thus improves the reliability and accuracy of the determination. This is the main argument for using multi-column chromatography with column switching for this particular purpose.

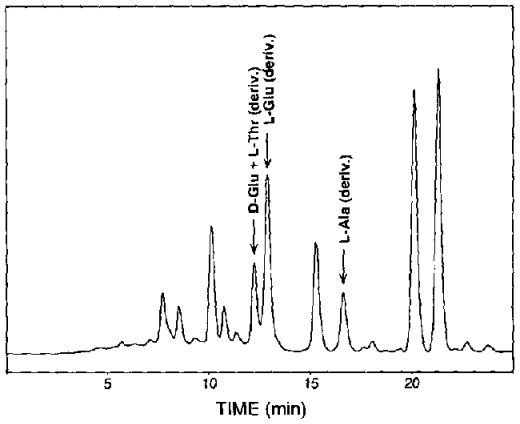

Fig. 4. Chromatogram of the same protein hydrolysate as in Figs. 2 and 3 after derivatization with the (+)FLEC reagent [11]. Chromatographic conditions: column, DeltaPak C_{18}, 100 Å, 150 mm × 4.6 mm I.D. (Waters); mobile phase, aqueous buffer (0.03 *M* sodium acetate, pH 4.35) with 25% (v/v) tetrahydrofuran; flow-rate, 1.0 ml/min; temperature, ambient; excitation wavelength, 260 nm; emission wavelength, 310 nm.

Fig. 4 shows the chromatogram of the same prepurified protein hydrolysate analysed in Fig. 3, using single-column chromatography after derivatization with the (+)FLEC reagent. The peaks of D-Glu and L-Thr coincide. The numerous other peaks in the chromatogram show that there is a high probability of peak overlap, even if there would be no apparent coincidence of peaks. This becomes particularly important when dealing with low percentages of D-amino acids in the sample.

The proposed method is not limited to the determination of the enantiomeric composition of Ala and Glu, but can also be used for the determination of the enantiomeric excess of several other dansylated amino acids, *e.g.* Dns-Thr, Dns-Leu, Dns-Val. In these instances the elution protocol for the first column can readily be adapted in such a way that the dansylated amino acids of

[a] The biological significance of our findings will be discussed elsewhere.

interest are separated [14]. Whereas most of the amino acids mentioned can be separated into enantiomers when a chiral system is used with bonded β-cyclodextrin selectors, a satisfactory separation of the enantiomers of Dns-Ala has not yet been obtained on a bonded β-cyclodextrin phase.

Precautions should be taken when working with concentrated solutions of β-cyclodextrin: its solubility in water–ethanol is limited, but can be enhanced by adding urea. When β-cyclodextrin is used in rather high concentrations, the chromatographic system, including the pump and detector, should be rinsed carefully before allowing a longer stand-by period.

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