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Two-dimensional high-performance liquid chromatographic system for the determination of enantiomeric excess in complex amino acid mixtures

Single amino acid analysis

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ABSTRACT

An HPLC system that allows the determination of the enantiomeric composition of complex mixtures of amino acids such as those occurring in biological fluids (*e.g.*, serum, cerebrospinal fluid) and foods is described. D- and L-amino acids (including proline) can be determined. First, amino acid separation is achieved by means of an ion-exchange column by elution with a lithium chloride–lithium citrate buffer. Each peak corresponding to an individual amino acid can be switched to a reversed-phase column (C_{18}) and eluted with an aqueous solution containing chiral copper(II) complexes which perform chiral discrimination by a ligand-exchange mechanism. The method is very flexible as several chiral selectors and different types of detection (*e.g.*, UV, fluorescence) can be used. Moreover, it avoids unnecessary overrunning of the chiral system with the whole mixture, by switching only the peaks under investigation. It is possible to evaluate D-amino acids up to a 0.1% D to D + L ratio in the nanomolar range. Postcolumn derivatization with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole and fluorimetric detection were utilized for proline and hydroxyproline and with *o*-phthaldehyde for the other amino acids.

INTRODUCTION

The presence of D-amino acids in nature has become an increasingly fascinating subject. The biological role of such unusual "oddities", not only in prokariotes and in eukariotes [1], but also in higher organisms such as insects, sharks, guinea pigs and marine invertebrates [2,3], is not known.

Recently, chiral separations of modified and unmodified amino acids have been achieved by HPLC, utilizing either chiral derivatizing agents [4–7] or chiral complexes added to the mobile phase [8,9]. Unfortunately, the methods are not readily applicable to complex mixtures, such as those present in natural systems, biological fluids, foods and beverages, because both chemoselectivity and stereoselectivity have to be taken into consideration. Moreover, although the biological matrices are in most instances extracted with ethereal solvents and eluted through ion-exchange resins before the analysis, nevertheless, amino alcohols, biogenic amines, dipeptides and oligopeptides are often present together with the desired amino acids, giving rise to unknown peaks that interfere in the chromatograms.

Despite the possibility of cross-checking the results by using different methods (GC, pre- and post-column derivatization in HPLC) [10,11], it is difficult to obtain very reliable and reproducible determinations of the enantiomeric ratio of

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amino acids in very complex and diluted mixtures, such as serum or cerebrospinal fluid (CSF). This may lead to undesirable controversies such as that concerning the racemization of proline in connection with microwave heating [12–15].

In the past, we developed a good method for the separation of D,L-dansylamino (Dns-amino) acids in HPLC by using either copper(II) complexes of L-amino acid amides [16] or of diaminodiamido-type ligands [17]. With the former complexes we were able to separate a mixture of fifteen D,L-Dns-amino acids by utilizing a gradient system in 1.5 h.

However, when trying to determine the D/L ratio with the same system, we realized that D- and L-enantiomers of all Dns-amino acids give different fluorescent responses. The phenomenon was thoroughly investigated by fluorescence experiments in aqueous solution [18], which led to the discovery of the enantioselective fluorescence quenching of D- and L-Dns-amino acids by L-amino acid amidate copper(II) complexes. This, on the one hand, allowed several clues to be obtained on the mechanism of chiral discrimination by a ligand-exchange mechanism, and on the other it caused concern regarding the use of the method for the determination of the D/L ratio. Subsequently, we advised caution when using copper(II) complexes in the eluent for the determination of Dns-amino acids.

A system proposed by Tapuhi *et al.* [19] involved the separation of Dns-amino acids on an achiral C_{18} column, switching of a single peak to another C_8 column eluted with an Ni(II) complex of L-prolyl-*n*-octylamide. Ni(II) is also known to be a fluorescence quencher of Dns-amino acids and peptides [20]. Hence also in this case enantioselective fluorescence quenching could be possible, impairing the determination of the D/L ratio.

For all these reasons, we decided to develop a method for the separation of unmodified amino acids in complex mixtures, avoiding prederivatization procedures and work-up of the biological samples.

We report here a method that allows the problem to be addressed in a sound and reliable way, a two-dimensional HPLC procedure that utilizes two different separation methods: first the achiral separation of unmodified amino acids on a cation-exchange column, switching of the amino acid under investigation to a reversed-phase column and chiral discrimination by elution with a chiral copper(II) complex. Several copper(II) complexes can be used according to the amino acid to be separated.

The advantage over the reported methods, which operated "in series", is that in this way we measure only the single amino acid under investigation, avoiding overusage and spoilage of expensive chiral columns, thus obtaining a "clean" separation and saving time. By using the present method, we were able to demonstrate that, under normal microwave heating conditions, racemization does not occur in either proteins or the free amino acid pool [21].

EXPERIMENTAL

Reagents

o-Phthalaldehyde (OPA) and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) were obtained from Fluka (Buchs, Switzerland) and Pierce (Rockford, IL, USA). D,L and D- and L-amino acids were obtained from Sigma (St. Louis, MO, USA) and Pierce. Acetonitrile (LC grade), methanol (LC grade), copper(II) acetate and sodium acetate were obtained from Carlo Erba (Milan, Italy). Lithium hydroxide, lithium chloride, citric acid, phenol, boric acid, 2-mercaptoethanol, sodium hypochlorite and hydrochloric acid were obtained from Fluka. Water (HPLC grade) was produced in our laboratory utilizing an Alpha-Q system (Millipore).

The chiral selectors used (N^2,N^2-dimethyl-L-phenylalaninamide (Me_2Phe-A) [22], N^2-methyl-L-phenylalaninamide (MePhe-A) [23] and bis (L,L-N,N′-dimethylphenylalanyl)ethane (Me_2-Phe-NN-2) [21,24]) were synthesized in our laboratory. Their copper(II) complexes were characterized [22–25] and their ability to perform chiral discrimination in reversed-phase HPLC [23] was examined.

Equipment

The chromatographic system utilized is shown in Fig. 1. It is composed of two parts: a common

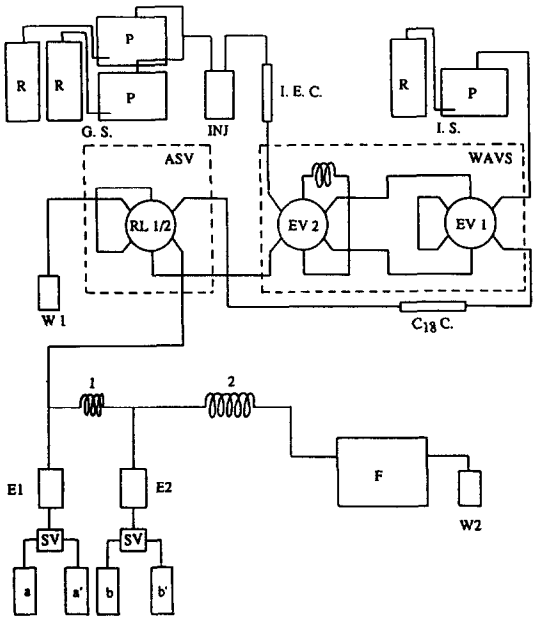

Fig. 1. Scheme of the chromatographic system for single amino acid analysis. G.S. = gradient system; I.S. = isocratic system; I.E.C. = ion-exchange column; $C_{18}C.$ = C_{18} reversed-phase column, EV 1 and EV 2 = events 1 and 2; F = fluorescence detector; 1 and 2 = reaction coils 1 and 2, E 1 and E 2 = Eldex pumps 1 and 2; ASV = automated switching valve; WAVS = Waters automated valve system; R = reservoir; P = pump; SV = switching valve; a and b = derivatizing reagents for ion-exchange analysis; a' and b' = derivatizing reagents for enantiomeric separation.

ion-exchange apparatus for the separation of amino acids and a reversed-phase column for the chiral separation. The two chromatographic systems are interconnected through a Waters WAVS valve and a Waters automated switching valve. The system is controlled by a Maxima 820 chromatography workstation. The gradient system (G.S.) for the ion-exchange separation of amino acids consists of two Waters Model 510 pumps. The samples are injected utilizing a WISP 712 automatic sample processor (Waters). The mobile phase for the isocratic system, responsible for the enantiomeric separations, is delivered using another Waters Model 510 pump. The eluate is mixed, in two different T-pieces, with the derivatizing agents necessary for the analysis.

The postcolumn derivatization differs according to the analyte: a single treatment, using OPA, for all amino acids and a two-stage treatment for proline and hydroxyproline. The derivatizing agents are delivered using one or two (in series) single plunger pumps (Eldex, E1 and E2). For proline, two manual switching valves are utilized to change the derivatizing eluents necessary to effect detection during the ion-exchange separation (reagent a, borate buffer containing sodium hypochlorite; reagent b, borate buffer containing OPA) and during the reversed-phase enantiodiscrimination (reagent a', borate buffer containing EDTA; reagent b', NBD-Cl in ethanol). Both the reaction coil 1 (stainless steel, 1000×0.5 mm I.D.) and the reaction coil 2 (stainless steel, 4000×0.5 mm I.D.) are kept at 55°C. The fluorescence intensity of the effluent is measured using a Waters Model 470 spectrofluorimeter (for OPA derivatives, $\lambda_{exc.} = 330$ nm, $\lambda_{em.} = 440$ nm; for NBD-Cl derivatives, $\lambda_{exc.} = 465$ nm, $\lambda_{em.} = 525$ nm).

Columns

Amino acids are separated on an Interaction amino acid analysis column (12×0.46 cm I.D.), a sulphonated polystyrene–divinylbenzene copolymer in the lithium form, spherical particles with a diameter of 6 ± 0.5 μm. The reversed-phase column used for the enantiomeric separation is a Spherisorb ODS-2, 3 μm (15×0.46 cm I.D.). The ion-exchange column is maintained at 45°C and the reversed-phase column at 25 or 35°C depending on the amino acid to be measured.

Eluents for ion exchange (flow-rate 0.4 ml/min)

Eluent A is $LiOH \cdot H_2O$ (2.1 g), LiCl (6.0 g), citric acid (9.6 g) and phenol (1.0 g), diluted to 1 l and adjusted to pH 2.87 with HCl.

Eluent B is $LiOH \cdot H_2O$ (2.1 g), LiCl (65.7 g), phenol (1.0 g) and boric acid (1.0 g), diluted to 1 l and adjusted to pH 9.20 with LiOH.

Eluents for enantiomeric separation (flow-rate 0.3–2.0 ml/min)

Eluent C is Me_2Phe-A or MePhe-A. The ligand (2 m*M*) and copper(II) acetate (1 m*M*) are added to 1 l of water containing 0.3 *M* sodium acetate and adjusted to pH 6.35 with acetic acid.

Eluent D is Me_2Phe-NN-2 (0.45 m*M*) and copper(II) acetate (0.45 m*M*) added to 1 l of water containing sodium acetate (4.2 g), LiCl (1.8 g) and $LiOH \cdot H_2O$ (0.63 g) and adjusted to pH 6.5 with acetic acid. For apolar amino acids 5% CH_3CN is added.

Post-column derivatization with OPA (for ion exchange and for enantiomeric separation): one Eldex pump

EDTA (2.0 g) is dissolved in 1 l of 0.3 *M* borate buffer (pH 10, adjusted with KOH) and mixed with OPA (0.8 g) dissolved in 2-mercaptoethanol (4 ml) (flow-rate 0.6 ml/min).

Derivatization of D,L-proline

The derivatization of D,L-proline is achieved by the following procedure. For ion-exchange analysis (two Eldex pumps), (1) the first reagent (a) is 0.1% sodium hypochlorite (chlorine concentration 10%) dissolved in 1 l of 0.4 *M* borate buffer (pH 10, adjusted with KOH) (flow-rate 0.6 ml/min) and (2) the second reagent (b) is OPA (0.8 g) dissolved in 2-mercaptoethanol (4 ml), added to 1 l of 0.3 *M* borate buffer (pH 10, adjusted with KOH) (flow-rate 0.6 ml/min).

For enantiomeric separation (two Eldex pumps), (1) the first reagent (a′) is EDTA (2 g) dissolved in 1 l of a mixture (1:1) of ethanol and 0.05 *M* borate buffer (pH 9.5, adjusted with KOH) (flow-rate 0.6 ml/min) and (2) the second reagent (b′) is NBD-Cl (1 g) dissolved in ethanol (500 ml) (flow-rate 0.6 ml/min).

RESULTS AND DISCUSSION

In order to achieve the enantiomeric analysis of each individual amino acid in complex biological mixtures, we modified our HPLC system according to the scheme shown in Fig. 1. The aim was to avoid cross-interference with dipeptides, oligopeptides, amino alcohols and biogenic amines.

The system is based on a preliminary ion-exchange achiral separation of the mixture with a gradient system and a subsequent chiral separation on an achiral reversed-phase column and elution with chiral copper(II) complexes added to the eluent. The selectors utilized are mainly chosen among the series described elsewhere [23], in particular the copper(II) complexes of MePhe-A, Me_2Phe-A and Me_2Phe-NN-2. When the peak corresponding to the amino acid to be investigated, during the ion exchange run, arrives at the loop present in EV2, the configuration of Event 2 (EV2) is changed in order to block the unmodified amino acid in the loop. Immediately afterwards, the CSV valve is turned to the eluent containing the chiral selector with the derivatizing agent, thus discharging the ion-exchange buffered eluents into waste 1 and simultaneously allowing the reaction coils to be washed with the new mixture. When the system is equilibrated, by switching Event 1 (EV1), the amino acid blocked in the loop (EV2) is injected into the reversed-phase column responsible for the enantiomeric separations (Fig. 2). The same derivatizing agent is used for the two different analyses (borate buffer, containing OPA and EDTA).

In order to detect the enantiomers of imino acids such as proline or hydroxyproline, a different derivatization agent is used. As described under Experimental, two different single-plunger pumps are necessary: the first is used to detect proline in the ion-exchange analysis and the second to derivatize, after the enantiomeric separation, the same amino acid with a fluorescent reagent able to react with secondary amines.

On the basis of the considerations reported elsewhere [23], it is reasonable to assume that enantiomeric recognition occurs on the column where the initial complexes are adsorbed. Therefore, it is necessary to allow the eluent containing the copper complex to flow through the column for several minutes (120 min at 1 ml/min, 30 column volumes).

The elution order of the enantiomers is L < D for polar and D < L for apolar amino acids when using copper(II) complexes of Me_2Phe-NN-2, MePhe-A and Me_2Phe-A as chiral selectors. Further control can be effectively performed if one wishes, as is advisable, to have the less abundant enantiomer eluted before the more abundant enantiomer, by using the selector with opposite configuration (*e.g.*, D-MePhe-A). In this event D-Asp and D-Glu are eluted first. How-

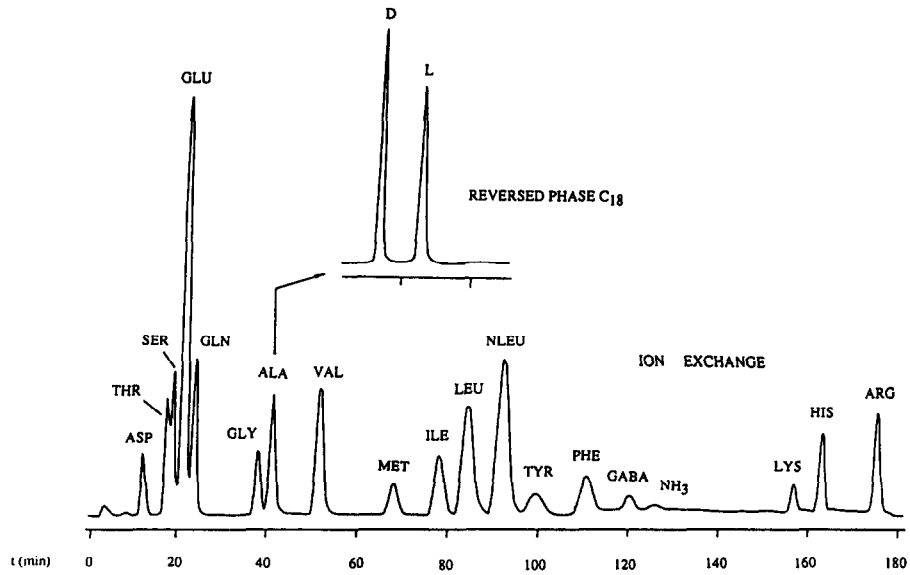

Fig. 2. Example of a single amino acid analysis. Lower trace: Separation of amino acids by ion exchange. Column, Interaction, lithium form (6 μm) (12 × 0.46 cm I.D.); temperature, 45°C; eluents A and B, see Experimental; flow-rate, 0.4 ml/min. Inset: Enantiomeric separation on a Spherisorb ODS-2 column (3 μm) (15 × 0.46 cm I.D.); temperature, 25°C; eluent, MePhe-A (2 m*M*)–copper acetate (1 m*M*)–sodium acetate (0.3 *M*) (pH 6.35); flow-rate, 0.5 ml/min.

ever, in most instances examined so far this was not necessary, because the separation factors (α) are sufficiently high to cause no concern about the latter enantiomer being "carried along" by the former. Me_2Phe-A is preferentially used to perform chiral discrimination of proline (Fig. 3) and Me_2Phe-NN-2 for polar and apolar amino acids (Fig. 4).

After the enantiomeric analysis, the chiral eluent can be recovered in the following way. Copper is precipitated as sulphide with gaseous hydrogen sulphide in acidic solution and then,

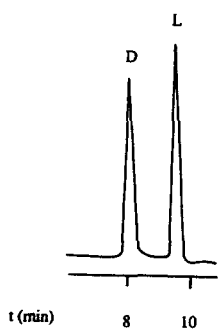

Fig. 3. Enantiomeric separation of D,L-Pro. Eluent, Me_2-Phe-A (2 m*M*)–copper acetate (1 m*M*)–sodium acetate (0.3 *M*) (pH 6.35); column, Spherisorb ODS-2 (3 μm) (15 × 0.46 cm I.D.); room temperature; flow-rate, 0.5 ml/min; fluorescence detection (postcolumn derivatization with NBD-Cl).

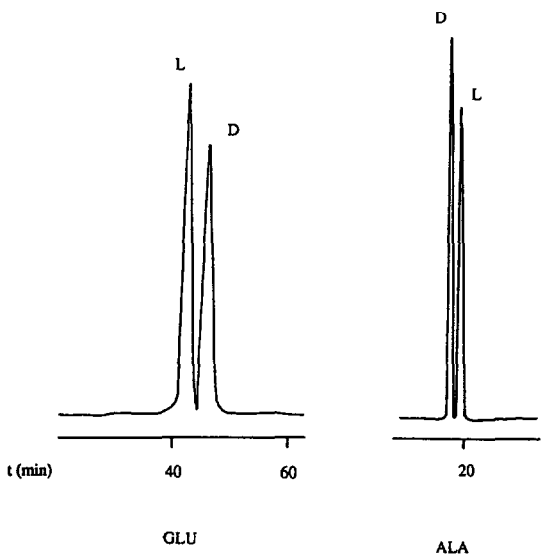

Fig. 4. Enantiomeric discrimination of polar and apolar amino acids. Eluent, Me_2-Phe-NN-2 (0.5 m*M*)–copper acetate (0.5 m*M*)–sodium acetate (0.05 *M*) (pH 6.5); column, Spherisorb ODS-2 (3 μm) (15 × 0.46 cm I.D.); room temperature; flow-rate, 0.5 ml/min; fluorescence detection (postcolumn derivatization with OPA).

after filtration, the aqueous solution is extracted with chloroform; sodium hydroxide is added to give basic conditions and the ligand is extracted with chloroform. The chloroform extracts are dried and the ligand is recrystallized twice from chloroform–diethyl ether. The recovered chiral ligand (90% of the total used) can be reutilized without loss of its discriminating ability.

CONCLUSIONS

Using the chromatographic method described here, it is possible to obtain optimum enantiomeric discrimination of amino and imino acids in complex mixtures, avoiding the occurrence of the pitfalls that often arise with biological matrices. The described apparatus can be totally automated and computer assisted to perform automated on-line determinations of D- and L-amino acids.

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