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Note

Enantiomeric analysis of a mixture of the common protein amino acids as their Dns derivatives

Single-analysis reversed-phase high-performance liquid chromatographic procedure using a chiral mobile phase additive

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Enantiomeric analysis of amino acids is important in many different fields, including asymmetric synthesis in organic chemistry, peptide synthesis, amino acid biogeochemistry, food processing and degradation processes associated with long-lived proteins in humans[1–4]. It has been severely restricted by the analytical difficulties involved. One reason for this is that no sensitive and quantitative instrument for enantiomeric analysis of amino acids is available that can also be automated in a manner comparable to the analysis of L-amino acids, using a dedicated conventional amino acid analyzer. With this in mind, and by making use of a new approach to enantiomeric analysis in which the chiral phase is added directly to the eluent solvents[5–7], we have developed a method for the analysis of almost all the D- and L-amino acids present in a protein hydrolysate. They are separated as their fluorescent 5-dimethylaminonaphthalene-1-sulphonyl (Dns) derivatives in one analysis, using reversed-phase liquid chromatography with N,N-di-*n*-propyl-L-alanine (L-DPA) and cupric acetate as the chiral additive. The method is quantitative, sensitive, fairly rapid (about 1.5 h) and can be adapted for routine, automated analysis, analogous to that used for L-amino acids.

Earlier studies have shown that Dns-L-amino acids can be separated by reversed-phase liquid chromatography[8–11]. The separation of the enantiomers of Dns-amino acids with chiral additives has also been demonstrated[12–16]. A chiral separation of all the underivatized amino acids in a protein hydrolysate has been reported by Weinstein *et al.*[17]. This involves the initial separation of the amino acids into three mixtures which are then each separated into their constituent enantiomers. This method cannot, however, easily be automated.

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MATERIALS AND METHODS

Reagents

Dns chloride (Dns-Cl) was purchased from Sigma (St. Louis, MO, U.S.A.). L-DPA was prepared according to the method of Bowman and Stroud[18], as modified by Weinstein *et al.*[17]. The compound was further purified, as follows: a solution containing 32 m*M* L-DPA dissolved in about 20 ml of water was passed through a glass column containing (2 × 1 cm) C_{18} reversed phase (40–63 μm) prepared according to the method of Hemetsberger *et al.*[19]. After discarding the first few drops, the eluate was collected, the column was washed with 5 ml water and the solution diluted to 100 ml to give a final concentration of 0.32 *M*. Smaller quantities of L-DPA can be purified with a disposable Waters Assoc. Sep-Pak C_{18} cartridge. This stock solution can be preserved for months without bacterial growth by adding a few drops of dilute cupric acetate solution and keeping it in a refrigerator. L-DPA will soon be available from J. T. Baker (Phillipsburg, NJ, U.S.A.).

All the solvents and chemical reagents used for preparing buffers were HPLC grade or analytical grade, respectively.

Chromatographic apparatus and conditions

A high-performance liquid chromatograph with an Aminco fluorescence detector using standard filters (excitation filter, 340 nm; emission filter, 425 nm long pass), an Eldex pump (Model A-30-S), a Rheodyne injector (Model 7125) and a simple low-cost gradient programmer[20] was used. A reversed-phase column (24 cm × 4.6 mm I.D.) laboratory-packed with Nucleosil C_{18} (5 μm) (Macherey Nagel, Düren, F.R.G.) was equilibrated with buffer (A) comprising 0.3 *M* sodium acetate and 23.5% (v/v) acetonitrile adjusted to pH 7.00 with glacial acetic acid. A 5-ml volume of chiral complex, prepared by mixing equal volumes of 0.32 *M* L-DPA and 0.16 *M* cupric acetate, was added to 400 ml of buffer solution to form a final concentration of 2 m*M* L-DPA and 1 m*M* cupric acetate. The second buffer (B) comprised 0.3 *M* sodium acetate and 40% (v/v) acetonitrile adjusted to pH 7.00 with glacial acetic acid. Chiral complex was added in the same proportions as for buffer A. The program used for separating the Dns-D,L-amino acids is shown in Table I. When using the low pressure gradient maker, care must be taken to maintain both buffers under equal hydrostatic pressure. The gradient program was initiated 20 min after sample injection. The flow-rate was 0.8 ml/min. Buffer B was pumped until the last peak (di-Dns-L-Lys) was eluted. Reequilibration with buffer A was achieved in 30 min.

Preparation of Dns-amino acids

An equimolar mixture of D,L-amino acids was prepared. Note that only L-isoleucine was included, its naturally occurring diastereoisomer, D-*allo*-isoleucine, being determined separately. D,L-Cysteine was not included in the mixture. Dns derivatization was carried out either by the method of Zanetta *et al.*[21] or by that of Tapuhi *et al.*[22], with similar results. One convenient routine procedure used by us, following that of Tapuhi *et al.*[22], is to mix 2.0 ml of 0.04 *M* Li_2CO_3 buffer (pH 9.5 with hydrochloric acid) containing 0.5 μ*M* of each α-amino acid with 1 ml Dns-Cl in acetonitrile (3.3 mg/ml), at room temperature for 2 h. Most of the Dns acid

TABLE I

GRADIENT PROGRAMS FOR SEPARATION OF Dns-AMINO ACIDS

A, Conditions used in Fig. 1; B, in the presence of large amounts of Dns-OH.

*Program A**		*Program B***	
Time after injection (min)	*Acetonitrile concentration (%, v/v)*	*Time after injection (min)*	*Acetonitrile concentration (%, v/v)*
0	23.5	0	14.3
20	25.1	10	16.2
30	26.8	20	18.0
40	28.5	25	20.7
50	31.7	30	23.5
60	35.6	35	26.2
65	40.0	40	29.0
		45	31.7
		55	34.5
		60	39.1
		65	40.0

* Column equilibrated with buffer A (23.5% acetonitrile) 30 min before injection; flow-rate 0.8 ml/min. For details see Materials and methods.

** Eluent: 0.25 *M* sodium acetate, water–acetonitrile buffer pH 7.0, 4 m*M* L-DPA, 2 m*M* cupric acetate, column equilibrated with buffer containing 12.5% acetonitrile 20 min before injection; flow-rate 0.4 ml/min.

(Dns-OH) was then separated from the Dns-amino acids, by loading 20 μl of the solution onto a small column (3 × 0.2 cm I.D.) packed with silica (40–63 μm). Immediately following the application of the sample (using a syringe), the column was eluted with methylene chloride–diethyl ether–acetic acid (40:10:20). The Dns-amino acids are eluted in the first 0.4 ml, whereas the Dns-OH requires a much larger volume. The separation can be monitored visually under UV light: the Dns-OH has a blue fluorescence, whereas the Dns-amino acids are yellow-green. The sample solution was evaporated to dryness in a stream of nitrogen and dissolved in buffer B for injection.

RESULTS

The separation of a standard mixture of Dns-D,L-amino acids is shown in Fig. 1. Almost all the enantiomers are completely or partially separated from each other. Dns-D-arginine (not shown) is closely associated with the dansylic acid peak. The enantiomers of proline are not separated using this chiral complex. In this chromatogram they almost co-elute with Dns-glycine. If necessary, a slight delay in the onset of the gradient will result in the separation of Dns-glycine and Dns-proline. Although histidine was present in the standard amino acid mixture, it apparently has a relatively weak fluorescence in these solvents and was not identified in the chromatogram. The elution positions of Dns-D-*allo*-isoleucine, Dns-D-tyrosine and Dns-L-tyrosine are also shown in Fig. 1. D-*allo*-Isoleucine is the naturally formed isomer of L-isoleucine. Tyrosine is oxidized in the presence of copper and is rapidly

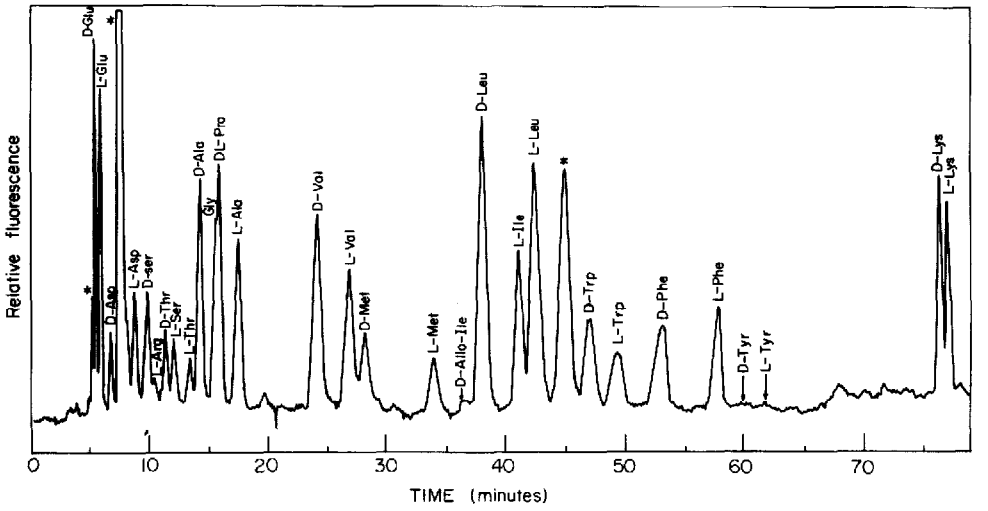

Fig. 1. Chromatogram showing the separation of almost all the amino acids present in a protein hydrolysate analysed as their fluorescent Dns derivatives. Dns derivatization side-products are indicated by asterisks. The large peak appearing after 7 min is Dns-OH and the peak at 45 min is Dns-amide. Arrows denote the elution positions of compounds not present in the mixture (Dns-D-*allo*-isoleucine), or decomposed during the analysis (Dns-D- and Dns-L-tyrosine). Chromatographic conditions are given in Table I, program A.

lost during the separation procedure. In equimolar standards, only trace amounts of the original tyrosine residues were detected. The enantiomers of tryptophan, partially decomposed in an acid hydrolysate, are also well separated. Thus, thirteen enantiomeric pairs of amino acids are well separated from each other and from the three major Dns-Cl breakdown products.

We noted during this study that over a period of time the standard solutions of the Dns-amino acids gave different relative peak areas for some enantiomeric pairs. The Dns-L-serine and Dns-L-threonine peaks were particularly susceptible. The preference for the L-enantiomers suggests that this is due to degradation by microorganisms.

The Dns-D-amino acids are always eluted before the Dns-L-amino acids. By replacing L-DPA with D-DPA, the order of elution can easily be reversed to reveal peaks not well separated with the L-ligand. In analyses of natural samples, which often contain additional unknown peaks, this procedure is useful for confirming that a particular peak is homogeneous and not contaminated by some other compounds.

The removal of most of the excess of Dns-OH is essential for obtaining satisfactory analyses of Dns-D- and L-aspartic acid and Dns-D- and L-glutamic acid. The Dns-OH peak not only masks the aspartic acid peaks, but also changes the retention time of the peaks that are eluted in that region by saturating the column. A simple and convenient method is described for removing most of the Dns-OH. It should be noted, however, that the polar and acidic amino acids are eluted soon after the Dns-OH and the possibility exists that some of the peaks may be removed with the Dns-OH. If it is inconvenient to remove the excess of Dns-OH, then an alternative chromatographic procedure can be followed, which prolongs the analysis time con-

siderably and to some extent complicates the analysis of the peaks which have longer retention times. The solvent program is also given in Table I. If excess of Dns-OH is not removed, the original analytical conditions are satisfactory, starting with D-threonine or L-serine.

As the analytical conditions for separation vary between instruments and are particularly dependent on the quality of the column used, we have investigated, in some detail, the effect of varying different parameters of the eluent solutions on peak retention times. This information should facilitate the introduction of minor adjustments to the chromatographic conditions in order to achieve satisfactory separations when necessary.

The ionic strength of the eluting solutions strongly influences the retention times. By increasing the sodium acetate concentrations from 0.05 to 0.4 *M*, the retention times of some acidic and polar amino acids, for example, could be reduced by approximately 60–70%. The Dns-OH peak, however, is fairly insensitive to ionic strength changes. Thus, small variations in ionic strength can be exploited for improving the separation of the peaks that are eluted in the vicinity of the Dns-OH peak. An increase of the pH of the eluent solutions from 6.0 to 7.3 resulted in a marked increase in retention times of all the amino acids, as does an increase in the concentration of the complex of copper with L-DPA in the eluent solutions. We noted the latter effect in the concentration range 1.6–16 m*M* L-DPA. In contrast, varying the proportions of L-DPA to cupric acetate (from 2:1 to 4:0.8) did not appreciably affect the retention times or the resolution between enantiomeric pairs. The optimal proportions with respect to efficient usage of the chiral phase are 2:1. We have also examined the ability of various chiral ligands such as N,N-dimethylvaline, N,N-dimethylalanine and N,N-dimethylleucine to separate Dns-L- and D-amino acids. None of them is, however, as effective as L-DPA.

The above observations clearly show that the separations achieved are very sensitive to the buffer compositions. Precautions should therefore be taken to reduce buffer evaporation or change in pH which may occur as a result of equilibration with atmospheric carbon dioxide during the analysis, if reproducibility from one analysis to another is to be maintained.

DISCUSSION

The method described here is a convenient procedure for performing routine, automated enantiomeric analyses of amino acids. The derivatization of the samples with Dns-Cl prior to analysis and the removal of excess of Dns-OH are simple and rapid procedures. Pre-column derivatization has the important advantage of minimizing sample contamination during handling and analysis. Post-column derivatization and detection procedures, on the other hand, are limited by background contamination due to the presence in the eluent solutions of compounds containing amino groups. Furthermore, the fact that derivatized amino acids are fluorescent facilitates the analysis of natural samples which usually contain many non-amino acid molecules. Only those contaminating compounds, which contain groups that react with dansyl chloride, will potentially interfere with the analysis. Monitoring the eluent for UV absorption, at 254 nm, for example, is possible, as Dns-amino acids absorb very strongly at this wavelength. The disadvantages are that many other

compounds also absorb in this region, thus increasing the possibility of having contaminants which mask amino acid peaks. Furthermore, the chiral complex also absorbs at 254 nm and as a result the baseline fluctuates considerably.

The method we describe offers the option of separating all the amino acid enantiomers that are eluted before Dns-D-leucine under isocratic conditions, using buffer A, within 45 min after injection. A one-step switch to a second buffer containing approximately 32% (v/v) acetonitrile results in the separation of most of the remaining amino acids, except di-Dns-lysine which requires at least 40% (v/v) acetonitrile in the eluent solution. It is therefore possible that an automated procedure can be developed based on three different eluent solutions, thus alleviating the need for a gradient program.

Methods for enantiomeric analysis of amino acids by gas chromatography[23–25] have been available for some time. They are not as convenient for routine, automated analyses as is the liquid chromatographic method described here, because of the somewhat more cumbersome sample derivatization procedures required and the lower sensitivities of conventional detectors. Furthermore, the gas chromatographic method requires a dedicated chiral column, whereas the liquid chromatographic method employs a non-dedicated, widely available C_{18} reversed-phase column. In addition, with the high-performance liquid chromatographic procedure, the elution sequence of the enantiomers can easily be reversed by changing the chirality of the dipropylalanine ligand, thus providing a rapid and effective means of peak identification.

The need for an automated, routine method for enantiomeric amino acid analysis exists in many fields. For example, many biological tissues or even isolated proteins, peptides, hormones, antibiotics, etc., have not been carefully screened for the presence of D-amino acids. The analyses of amino acid enantiomers preserved in fossils is now widely used as a tool for resolving difficult stratigraphic problems by monitoring the increase in D-amino acid content of fossils with increasing age. The method we describe here can be used for the analysis of large numbers of samples. It requires the use of a conventional HPLC instrument and commercially available columns and reagents. Using this method, enantiomeric analyses of amino acids can be automated and hence performed routinely.

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