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SEPARATION OF UNDERIVATIZED DIPEPTIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON A WEAK ANION-EXCHANGE BONDED PHASE

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SUMMARY

Underivatized dipeptides have been separated by high-performance liquid chromatography on MicroPak AX-10 (a silica based, bonded phase, weak anion-exchanger) using mixtures of triethylammonium acetate buffer and acetonitrile as eluent. About fifty dipeptides were examined. Sequence isomeric and diastereoisomeric dipeptides were also tested. All pairs of sequence isomers examined were well separated. DL,DL-Dipeptides gave two peaks which were completely resolved. It was found out that these peaks correspond to mixtures of D,L- and L,D-isomers and of L,L- and D,D-isomers, respectively. Separation of oligomers into classes by chain length was also demonstrated.

The described method is sensitive, fast and gave excellent separations. Sharp symmetrical peaks were obtained. In all cases no double peaks were observed. Triethylammonium acetate buffer allows detection of free amino acids and peptides at wavelengths in the range 200–220 nm. Moreover, it is volatile and facilitates further direct investigation of separated compounds.

INTRODUCTION

One of the methods for sequencing of peptides involves their hydrolysis into dipeptides with dipeptidyl aminopeptidase I (DAP I)[1,2]. A further DAP I digestion of the peptide following removal of its N-terminal amino acid residue by a single Edman degradation[3] leads to a second, overlapping set of dipeptides. The identification of the released dipeptides in the two mixtures can be used to reconstruct the sequence of the peptides[2,4]. This, however, requires efficient methods to separate the digestion mixtures into their components.

Dipeptides have been analyzed by ion-exchange, paper and thin-layer chromatography[5–13]. Gas chromatography or combined gas chromatography–mass spectrometry has also been employed for separation and identification of dipeptides, but only after derivatization[14–22].

High-performance liquid chromatography (HPLC) has been widely used for

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separation of peptides[23–36]. Grushka and co-workers[25,26] reported the separation of some dipeptides containing aromatic amino acids on a tripeptide bonded stationary phase. A few pairs of sequence isomeric dipeptides have also been resolved by the above technique. Molnár and Horváth[27] obtained excellent separations of some dipeptides and other small peptides on non-polar stationary phases using either phosphate buffer or perchloric acid with acetonitrile as the gradient former. The separation of underivatized dipeptides on four different reversed phases has been described by Lundanes and Greibrokk[32]. The capacity factors of the separated compounds were measured, but no chromatograms were given. Nakamura *et al.*[34] separated some histidine containing dipeptides on a strong cation-exchanger.

In this paper, we describe the separation of underivatized dipeptides by HPLC on a silica based bonded phase weak anion-exchanger using mixtures of triethylammonium acetate buffer and acetonitrile as the eluent. About fifty dipeptides containing all kind of amino acids were examined. Separation of sequence isomeric dipeptides, resolution of diastereoisomers and fractionation of oligomers into classes by chain length was also undertaken.

Triethylammonium acetate buffer has been chosen as the eluent because it shows an excellent miscibility with acetonitrile[37], buffers well at the pH employed and is easily prepared from triethylamine and acetic acids[38,39]. Its major advantage is that it allows detection of eluted amino acids and peptides at wavelengths in the range of 200–220 nm because of its low absorption in that region. In addition, this buffer is readily removed from the eluted samples by freeze-drying[38]. This property of the eluent facilitates further isolation and analysis of the separated compounds.

EXPERIMENTAL

Apparatus

Separations were performed using a Varian Model 8500 liquid chromatograph (Varian, Walnut Creek, CA, U.S.A.) equipped with a high-pressure sampling valve (Valco, Houston, TX, U.S.A.). The column effluents were monitored by means of a variable wavelength detector (cell volume, 8 μl) (Schoeffel SF 770, Westwood, NJ, U.S.A.). Filtration of the solvents was carried out using a pyrex filter holder (filter pore size, 0.22 μm) (Millipore, Bedford, MA, U.S.A.). Samples were taken up in deionized and distilled water and filtered by means of a Swinney filter (Millipore). Sample size varied between about 50 and 100 ng of material injected. A guard column packed with a pellicular anion-exchanger (Whatman, Clifton, NJ, U.S.A.) was used between the sampling valve and the column. All separations were carried out on a 30 × 0.4 cm MicroPak AX-10 column (Varian) which is a difunctional weak anion-exchange bonded phase prepared on LiChrosorb Si-60 silica (10 μm). Prior to chromatography the column was equilibrated with 400 ml of 0.5 *M* triethylammonium acetate (pH 3.4). This column provides efficient separations with either aqueous buffer or aqueous buffer–organic solvent mobile phases and has successfully been used for separation of nucleosides, nucleotides and bases[40]. The separation and sequencing of pyrimidine deoxypentanucleotide sequence isomers has also been performed using this column[37].

Materials

Dipeptides were obtained from Sigma (St. Louis, MO, U.S.A.) and Research

Plus Labs. (Denville, NJ, U.S.A.). Triethylamine (Eastman Kodak, Rochester, NY, U.S.A.) was purified by refluxing for 3 h with 2,4-diaminophenol dihydrochloride followed by distillation[39]. A stock solution of 2 *M* triethylammonium acetate (pH 4.3) was prepared by addition of triethylamine to an acetic acid solution. Working solutions were prepared by dilution of the stock solution and titration with glacial acetic acid to the desired pH value. Buffer solutions were prepared using deionized, distilled and filtered water. Glass-distilled acetonitrile was purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) and used as supplied.

RESULTS AND DISCUSSION*

Dipeptides are generally not retained on MicroPak AX-10 if buffer solution is used as the eluent. Addition of the organic solvent, acetonitrile, to the mobile phase increases retention times of dipeptides and facilitates their separation. Even aliphatic dipeptides are retained on MicroPak AX-10 and give reasonable retention times under the conditions described in this paper. Gradient elution may also be used to elute dipeptides with higher retention times by increasing the percentage of the buffer solution in the eluent.

Fig. 1 shows the separation of a mixture of sixteen selected dipeptides on MicroPak AX-10 at 40°C by isocratic elution with a mixture of 32% 0.01 *M* triethylammonium acetate (pH 4.3) and 68% acetonitrile. The absorbance profile represents a resolution of 12 peaks. Dipeptides containing acidic amino acids Asp and Glu do not elute under the conditions described in Fig. 1. Such compounds can be eluted from the column by decreasing the pH of the mobile phase. Separation of a mixture of six dipeptides containing acidic amino acids was accomplished using a mixture of 60% 0.01 *M* triethylammonium acetate (pH 3.1) and 40% acetonitrile (Fig. 2).

Separation of sequence isomeric dipeptides

The resolution of the sequence isomeric dipeptides is very important for the sequencing of peptides. In this paper, twelve pairs of sequence isomeric dipeptides were examined. Their separation was demonstrated in two mixtures. Fig. 3 shows the separation of a mixture of six pairs by isocratic elution with 32% 0.01 *M* triethylammonium acetate (pH 4.3) and 68% acetonitrile at 40°C. Except DL-Leu-DL-Ala the dipeptides in this mixture were in L,L-configuration. DL-Leu-DL-Ala gave two peaks (peaks 1 and 4). The peak with the longer retention time most probably represents the L,L-configuration as discussed below in separation of diastereoisomers.

All pairs of sequence isomers were completely separated. The separation of the remaining six pairs is given in Fig. 4. Gradient elution was employed to elute L-Met-L-Ala (peak 11) and L-Ser-L-Ala (peak 12) in reasonable time. DL-Ala-DL-Ser gave two peaks (peaks 6 and 9). Peak 9 most probably represents the L,L-configuration (see below). With the exception of the pairs Gly-L-Leu, L-Leu-Gly and L-Ala-Gly, Gly-L-Ala, all pairs were completely separated. An acceptable resolution of these pairs, however, could be obtained (peaks 1,2 and 7,8, respectively).

The elution order of a pair of sequence isomeric dipeptides seems to obey

* Abbreviations for amino acids and peptides follow IUPAC-IUB recommendations (see *Biochem. J.*, 126 (1972) 773).

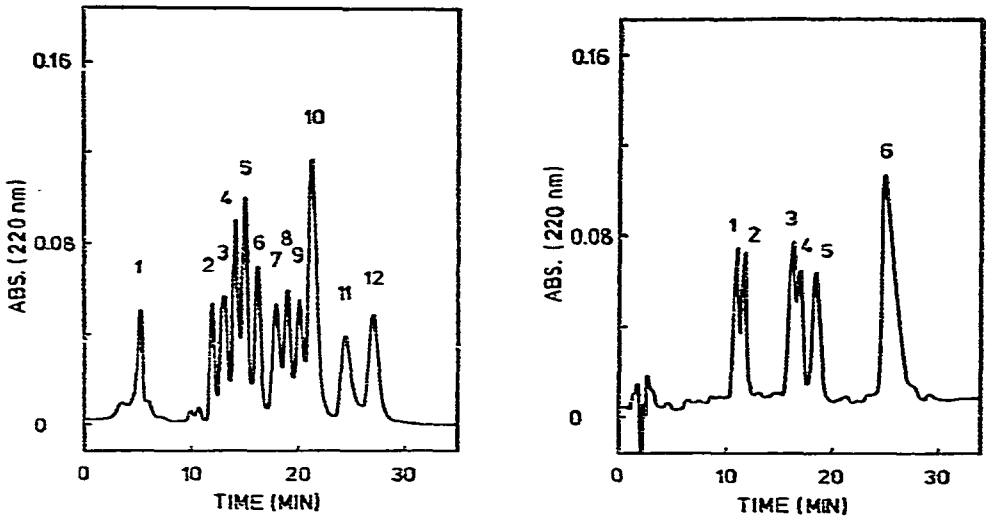

Fig. 1. Separation of some selected dipeptides. Column, MicroPak AX-10 (10 μm), 30 × 0.4 cm. Temperature, 40°C. Eluent, mixture of 32% 0.01 *M* triethylammonium acetate (pH 4.3) and 68% acetonitrile. Flow-rate, 1 ml/min. Peaks: 1 = L-Arg-L-Phe; 2 = L-Leu-L-Leu; 3 = Gly-L-Isoleu and L-Leu-L-Trp; 4 = L-Ala-L-Isoleu; 5 = L-Trp-Gly; 6 = L-Trp-L-Phe; 7 = L-Val-L-Val and L-Ala-L-His; 8 = L-Trp-L-Ala; 9 = L-Ala-L-Thr and L-Met-L-Met; 10 = Gly-Gly and L-Phe-L-Phe; 11 = L-Ser-L-Phe; 12 = L-Tyr-L-Tyr.

Fig. 2. Separation of some dipeptides containing acidic amino acids. Column as in Fig. 1. Temperature, 40°C. Eluent, mixture of 60% 0.01 *M* triethylammonium acetate (pH 3.1) and 40% acetonitrile. Flow-rate, 1.5 ml/min. Peaks: 1 = L-Ala-L-Glu; 2 = Gly-L-Glu; 3 = α-L-Glu-L-Ala; 4 = L-Ala-L-Asp; 5 = Gly-L-Asp; 6 = γ-L-Glu-L-Leu.

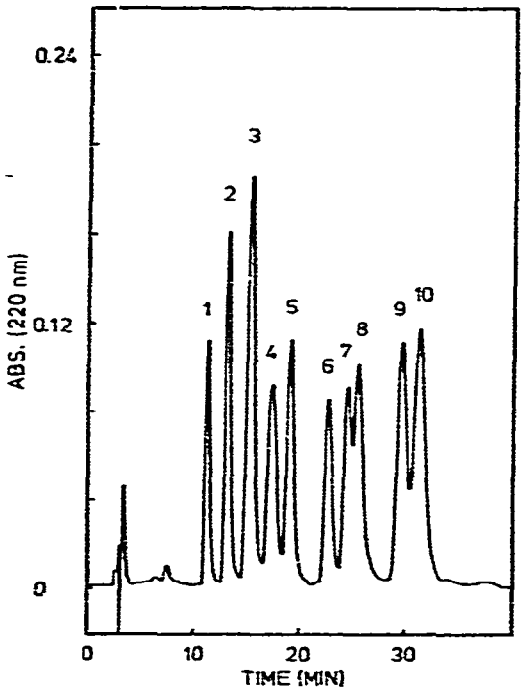

Fig. 3. Separation of sequence isomeric dipeptides. Column details as in Fig. 1. Peaks: 1 = DL-Leu-DL-Ala; 2 = Gly-L-Phe; 3 = L-Ala-L-Leu, Gly-L-Met and L-Ala-L-Phe; 4 = Gly-L-Tyr and DL-Leu-DL-Ala; 5 = L-Ala-L-Tyr; 6 = L-Phe-Gly; 7 = L-Tyr-Gly; 8 = L-Met-Gly; 9 = L-Phe-L-Ala; 10 = L-Tyr-L-Ala.

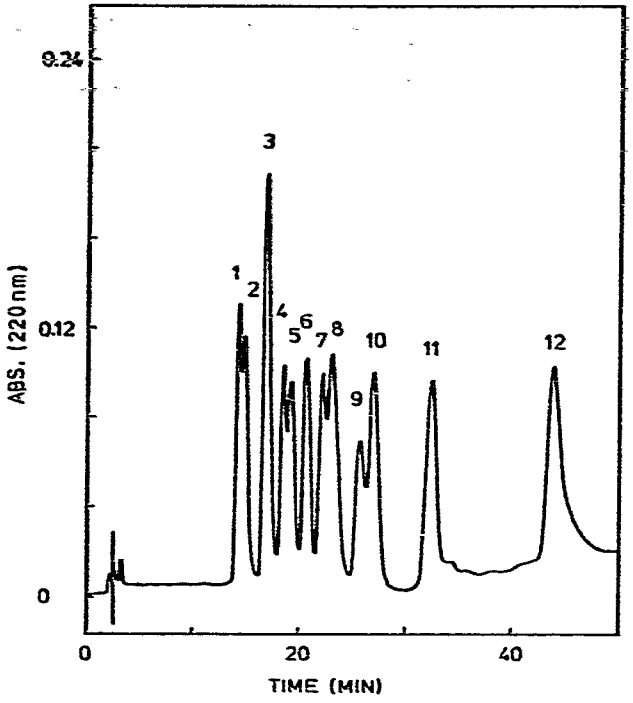

Fig. 4. Separation of sequence isomeric dipeptides. Column as in Fig. 1. Temperature, 50°C. Eluent: A, acetonitrile; B, 0.01 *M* triethylammonium acetate (pH 4.3), gradient program: step 1, isocratic with 70% A and 30% B for 25 min; step 2, linear gradient of 0.4% B per min. Flow-rate, 80 ml/h. Peaks: 1 = Gly-L-Leu; 2 = L-Leu-Gly; 3 = L-Ala-L-Met and Gly-L-Val; 4 = L-Val-L-Phe; 5 = L-Val-Gly; 6 = DL-Ala-DL-Ser; 7 = L-Ala-Gly; 8 = Gly-L-Ala; 9 = DL-Ala-DL-Ser; 10 = L-Phe-L-Val; 11 = L-Met-L-Ala; 12 = L-Ser-L-Ala.

certain rules. In the case of dipeptides containing only aliphatic amino acids, the dipeptide having a short side chain in the first amino acid elutes first: L-Ala-L-Leu < L-Leu-L-Ala (peaks 3,4); Gly-L-Met < L-Met-Gly (peaks 3,8) (Fig. 3); Gly-L-Leu < L-Leu-Gly (peaks 1,2); L-Ala-L-Met < L-Met-L-Ala (peaks 3,11); Gly-L-Val < L-Val-Gly (peaks 3,5); L-Ala-L-Ser < L-Ser-L-Ala (peaks 9,12) (Fig. 4).

Among seven examined pairs of this type there is one exception: L-Ala-Gly elutes faster than Gly-L-Ala (peaks 7 and 8 in Fig. 4).

In the case of dipeptides containing both alipathic and aromatic amino acids, the dipeptide having an aliphatic side chain in the first amino acid elutes first: Gly-L-Phe < L-Phe-Gly (peaks 2,6); L-Ala-L-Phe < L-Phe-L-Ala (peaks 3,9); Gly-L-Tyr < L-Tyr-Gly (peaks 4,7); L-Ala-L-Tyr < L-Tyr-L-Ala (peaks 5,10) (Fig. 3); L-Val-L-Phe < L-Phe-L-Val (peaks 4,10) Fig. 4).

These rules given above, of course, cannot be generalized without examining all possible pairs of sequence isomeric dipeptides.

Separation of diastereoisomers

Seven DL,DL-dipeptides were examined in order to find out whether the column used in this paper shows an ability to separate diastereoisomeric dipeptides.

The separation of these compounds were demonstrated in a mixture as shown in Fig. 5. All DL,DL-dipeptides with the exception of DL-Ala-DL-Phe gave two peaks which were completely resolved from each other. Apparently DL-Ala-DL-Phe do not contain the L,L-configuration since L-Ala-L-Phe shows a longer retention time (peak 6) than DL-Ala-DL-Phe (peak 2). Besides the complete resolution of two peaks from DL,DL-dipeptides this chromatogram also shows a nice separation of these compounds from each other.

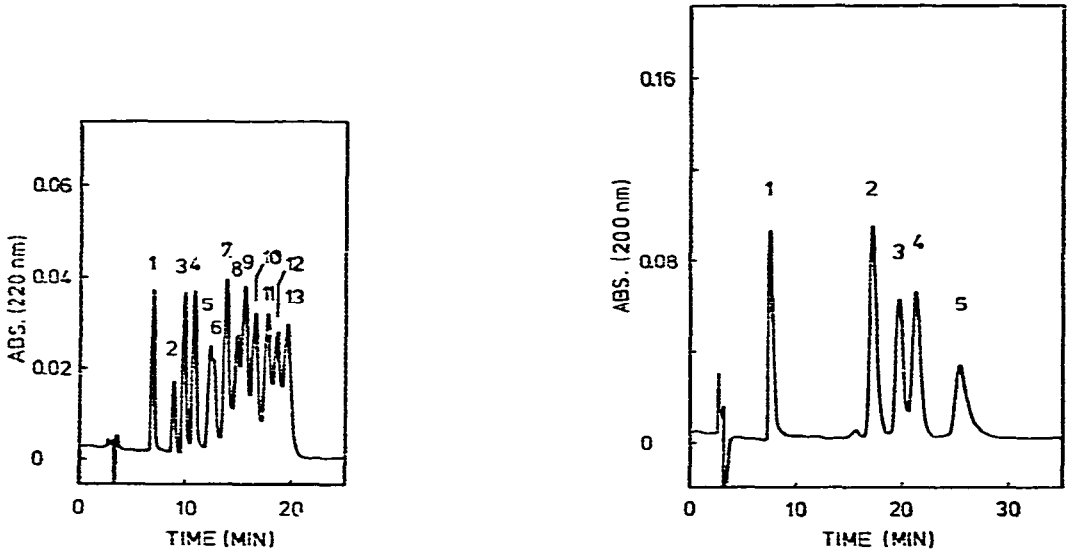

Fig. 5. Separation of diastereoisomeric dipeptides. Column as in Fig. 1. Temperature, 45°C. Eluent, mixture of 35% 0.01 *M* triethylammonium acetate (pH 4.3) and 65% acetonitrile. Flow-rate, 1 ml/min. Peaks: 1 = DL-Leu-DL-Phe; 2 = DL-Ala-DL-Phe; 3 = DL-Leu-DL-Ala; 4 = DL-Ala-DL-Val; 5 = DL-Leu-DL-Phe; 6 = L-Ala-L-Phe; 7 = DL-Ala-DL-Ala and DL-Ala-DL-Val; 8 = DL-Leu-DL-Ala; 9 = DL-Ala-DL-Ser; 10 = DL-Ala-DL-Asn; 11 = DL-Ala-DL-Ala; 12 = DL-Ala-DL-Ser; 13 = DL-Ala-DL-Asn.

Fig. 6. Separation of L-alanine oligomers. Column details as in Fig. 5. Peaks: 1 = L-Ala; 2 = L-Ala-L-Ala; 3 = tri-L-Ala; 4 = tetra-L-Ala; 5 = hexa-L-Ala.

Since all four diastereoisomers (D,L; L,D; L,L and D,D) were not available for the dipeptides investigated, the assignment of the peaks from DL,DL-dipeptides was based on the elution pattern of diastereoisomers of Ala-Ala, where all four configurations were available. In the Ala-Ala system the D,L- and L,D-isomers (peak 7) could be separated from D,D- and L,L-isomers (peak 11) but the two isomers in each group could not be separated from each other. Recently, Lundanes and Greibrokk[32] tested several diastereoisometric dipeptides on reversed-phase columns, and were able to separate L,L- and D,D-isomers from D,L- and L,D-isomers, but were also unable to separate the isomers within the two groups. They found that L,L- and D,D-isomers generally had lower retentions than the L,D- and D,L-isomers on reversed phases. Under our experimental conditions the elution order of these isomers is the reverse of that observed by Lundanes and Greibrokk[32] on reversed-phase columns.

Manning and Moore[5] who tested L,D- and L,L-isomers of twenty-one dipeptides

could separate L,D-isomers from L,L-isomers on a cation-exchanger using a conventional automatic amino acid analyzer. In all cases, L,D-isomers were eluted faster than L,L-isomers. This is the same elution order found in this paper, and is apparently characteristic of ion-exchange columns. Hence, the conclusion can be drawn that two peaks of each DL,DL-dipeptide in Fig. 5 (with the exception of DL-Ala-DL-Phe which gave only one peak) correspond to D,L- and L,D-isomers (shorter retention time) and to L,L- and D,D-isomers (longer retention time), respectively.

Separation of oligomers

L-Alanine oligomers have been used in order to find out whether the length of the peptide chain has a contribution to the retention of peptides under the conditions described in this paper. Fig. 6 shows the separation of L-alanine oligomers using a mixture of 35% 0.01 *M* triethylammonium acetate (pH 4.3) and 65% acetonitrile at 45°C. The monitoring wavelength was 200 nm in order to detect L-alanine. It can be seen that L-alanine is well retarded on the column under such conditions and elutes in a reasonable time. The oligomers are well separated from L-alanine and from each other. Penta-L-Ala was not available to us. It should be noticed that the difference between the retention time of L-alanine and those of its oligomers is much longer than that between the retention times of the oligomers.

The result obtained with Ala-oligomers suggests that the chain length has a contribution to the retention of peptides under the conditions used in this paper. However, further studies with other oligomers are required to support this finding.

Influence of temperature on retention and separation

The increase of the temperature generally causes an increase of the retention times under the conditions described above. In most cases the separation of a mixture could be improved by increasing the temperature to a certain limit starting at room temperature. For instance, L-Ala-L-Ser and L-Phe-L-Val (peaks 9 and 10, respectively, in Fig. 4) were not resolved at the temperatures below 50°C which was found to be the optimal temperature for this particular mixture. The other mixture of sequence isomeric dipeptides (Fig. 3), however, could be optimally separated at 40°C. This clearly shows that the temperature plays an important role in the separation of dipeptides under our experimental conditions.

CONCLUSIONS

The results obtained in this paper clearly demonstrate that underivatized dipeptides can be well separated by HPLC on a silica based, bonded phase, weak anion-exchanger using mixtures of triethylammonium acetate buffer and acetonitrile as eluent. The suggested method is also suitable for the separation of sequence isomeric and diastereoisomeric dipeptides. Results obtained with L-alanine and its oligomers suggest that free amino acids and small peptides can be also separated by this method. The excellent transparency of the used buffer for the UV light in the range 200–220 nm allows high sensitivity detection of both amino acids and peptides. The volatility of the buffer is another advantage when one wishes to isolate the separated compounds for further examinations.

Besides the percentages of the buffer and the organic solvent in the eluent the temperature also has a contribution to the retention. The optimal conditions of separation for a given mixture can be determined by varying these parameters.

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