Amino Acids (1991) 1: 331–337

Amino
Acids

Separation of D- and L-amino acids by ion exchange column chromatography in the form of alanyl dipeptides

J. Csapó, I. Tóth-Pósfai, and **ZS. Csapó-Kiss**

Faculty of Animal Science Kaposvár, Pannon Agricultural University, Kaposvár, Hungary

Summary. A method of ion exchange column chromatography was developed for the determination of D- and L-amino acids in the form of diastereomeric dipeptide. First the protein containing samples were hydrolyzed with 6 molar hydrochloric acid, then the single amino acids were separated in an LKB automated amino acid analyzer with the LKB fraction collector. Following lyophilization, the single amino acids were transformed into alanyl dipeptides with tertiary-butyloxycarbonil-L-alanine-N-hydroxy-succinimide (t-BOC-L-Ala-ONSu) active ester. The alanyl dipeptides were easily separated from one another and the initial amino acids. Determination of the D- and L-amino acids in this form is relatively accurate and reproducible but takes some time (33–38 min). Accuracy of the determination is satisfactory. The coefficient of variation amounts to 3–5%. The use of the method is suggested to laboratories having an amino acid analyzer and wish to determine D-and L-amino acids in synthetic-amino acids complements, peptides or natural materials.

Keywords: Amino acids – D- and L-amino acid determination – Diastereomeric dipeptides – Alanyl dipeptides – Ion exchange column chromatography

Introduction

Lately it became a more and more urgent requirement of our archeologist and stockbreeder colleagues to be able to separate and exactly determine D- and L-amino acids in different materials. In the last 25–30 years a great number of methods were developed by several authors to determine the age of fossils on the basis of amino acid racemization. The underlying principle of determination is the realization that L-amino acids forming the living organism, racemized after its death, are partly transformed into D-amino acids. With ruminant animal strains a part of the protein content of feed gets decomposed in the rumen and from the ammonia forming the microorganisms present build up their own protein, thus a part of the protein is transformed into microbial protein. A new method of following the decomposition of feed protein in the rumen is the

measurement of the D-alanine content, since D-alanine is formed only during microbiological protein synthesis, feed protein does not contain D-amino acids.

Several methods have been developed for the separation and determination of amino acid enantiomers. To study racemization of the purified amino acids polarimetry and various enzymatic processes were applied. Manning and Moore [4] described an ion exchange column chromatography technique for the separation of D- and L-amino acids and their quantitative determination. The method is suitable for the quantitative determination of trace amounts of D-amino acids beside L-amino acid. One of the most rapid methods for the separation of D- and L-amino acids is gas chromatography. Lately the use of liquid chromatography for separation of enantiomers is gaining ground.

By comparing the informations of the related literature [2, 3, 5, 6] to the possibilities in our laboratory we decided, taking into account the developments of recent years in peptide chemistry, to develop an ion exchange column chromatographic method for the separation of D-and L-amino acids in the form of diastereomeric dipeptides. In the development of the method we kept in view that the experiments described could be reproduced in a laboratory provided with an amino acid analyzer. The method should consist of simple steps, and suitable for routine analysis as given below:

- preparation of the sample;
- hydrolysis of the protein in the sample with hydrochloric acid;
- separation of the amino acids by ion exchange column chromatography;
- synthesis of the diastereomeric dipeptides;
- separation and determination of the diastereomeric dipeptides.

Experimental

Separation of the protein building amino acids

The raw protein content of the samples was determined with a Kjel-Foss 16200 rapid nitrogen analyzer and then depending on the raw protein content 100–1000 mg (equal to cca. 10–20 mg protein) material was hydrolyzed with 6 mol/l hydrochloric acid for 24 h. When hydrolysis was finished the hydrochloric acid was removed by lyophilization. The sample is ready for the determination of isoleucine and D-alloisoleucine by ion exchange column chromatography or by the same method for the separation of the protein building amino acids. Determination and separation have been carried out with LKB 4101 type amino acid analyzer and the connected LKB fraction collector. Conditions of separation were as follows:

Dimensions of the column	500 × 6 mm
Ion exchange resin	Chromex UA-8
Flow rate of buffer	80 $cm^3\ h^{-1}$
Flow rate of ninhydrin	40 $cm^3\ h^{-1}$
Column temperature	50°C for 60 min, the 70°C till the end of analysis

Buffer A: pH = 3.25; Na molarity = 0.2; 25 min
Buffer B: pH = 4.25; Na molarity = 0.2; 30 min
Buffer C: pH = 6.45; Na molarity = 1.2; 20 min
Sodium hydroxide: 0.4 mol; 15 min
Equilibration: Buffer A; 30 min

The test-tubes corresponding to individual amino acids were identified and lyophilized to the dry. Then the diastereomeric dipeptides were produced from the individual amino acids or from the mixture of several amino acids.

Synthesis of diastereomeric dipeptides

The synthetic amino acid or the residue separated with the amino acid analyzer and dried by lyophilization was dissolved in water to obtain a solution of 1–10% concentration for each amino acid. The pH of the solution was set at pH = 8 by adding 1–2 sodium hydrogen carbonate crystal, then the protected active ester of Ala dissolved in dioxane-water 1:1 was added in 2 to 2.5 times excess. The protected active ester of the alanine was synthesized as described by Bajusz [1]. The reaction mixture was shaken for 2 h in a shaking apparatus at room temperature, then lyophilized to dry. After drying the protective BOC group was cleaved off with 1 mol/l glacial acetic acid containing hydrochloric acid solution (reaction time 1 h), finally lyophilization was repeated. The alanyl dipeptides were then dissolved in citrate buffer of pH = 2.2. Appropriately diluted the solution was then applied to the ion exchange column of the amino acid analyzer to separate the diastereomeric dipeptides.

Separation of the diastereomeric dipeptides

The alanyl dipeptides were separated and determined in the LKB 4101 type automated amino acid analyzer. Conditions of separation do not differ from those described at separation of protein building amino acids.

Results and discussion

The most important step in our method is the synthesis and separation of diastereomeric dipeptides. The generally applied method for peptide synthesis in homogeneous solution is active ester condensation. Taking into consideration that the separation of the amino acids to be tested as well as the separation the diastereomeric dipeptides occurs in aqueous medium we chose of the active N-hydroxy-succinimide ester (ONSu). Next decision had to be taken on the group applied to protect the amino group of the acylating amino acid in the course of active ester condensation. We chose a tertiary butyloxycarbonyl group (BOC), partly because the BOC group is easy to build up, partly because after dipeptide synthesis the cleaving off the protecting group with trifluoroacetic or 1 mol/l glacial acetic acid containing hydrocloric acid can be simply carried out.

Subsequent to the selection of the protecting group and the active ester, the selection of the acylating amino acid had to be decided from among the amino acids building the protein. Since the acylating amino acid has to have an asymmetric center and connecting has to take the shortest possible time, our choice fell on alanine (Ala).

We synthesized to tertiary-butyloxycarbonyl-L-alanine-N-hydroxy-succinimide ester (t-BOC-L-Ala-ONSu), to determine the diastereomeric dipeptides of the acidic and neutral amino acids. The protected active ester of the amino acid was synthesized as described by Bajusz [1].

Subsequent to the synthesis of the active esters, diastereomeric dipeptides were prepared from crystallized amino acids (standards) or from the single amino acids separated in the amino acid analyzer.

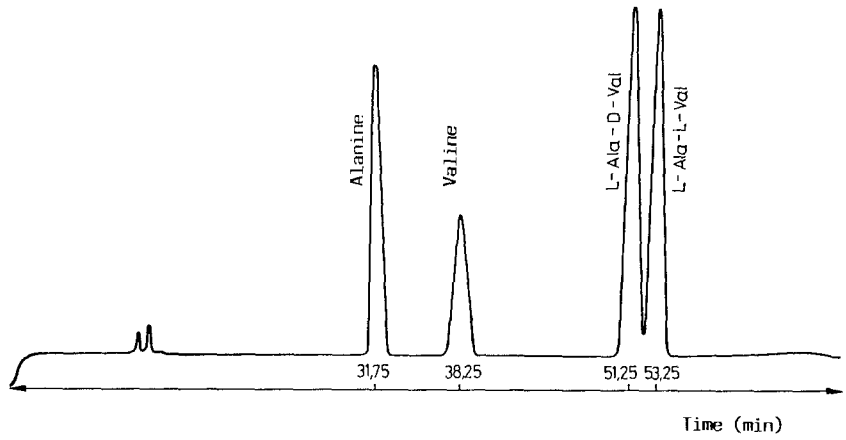

Fig. 1. Determination of D- and L-valine

The reactions are summarized as follows:

$$\text{t-BOC-L-Ala-ONSu} + H_2N\text{-}\underset{\underset{\text{COOH}}{|}}{\text{CH}}\text{-R} \xrightarrow[\text{2 hours}]{\text{pH=8,0}} \xrightarrow[\text{1 h}]{\text{1 mol HCl in glacial acetic acid}}$$

DL-amino acid

$$H_2N\text{-}\underset{\underset{CH_3}{|}}{\overset{\overset{H}{|}}{C}}\text{-CONH-}\underset{\underset{H}{|}}{\overset{\overset{R}{|}}{C}}\text{-COOH} + H_2N\text{-}\underset{\underset{CH_3}{|}}{\overset{\overset{H}{|}}{C}}\text{-CONH-}\underset{\underset{R}{|}}{\overset{\overset{H}{|}}{C}}\text{-COOH}$$

L-D dipeptide L-L dipeptide

The separation of L-L or D-L diastereomeric dipeptides formed from DL-Val and DL-Ile amino acids is shown in Figs. 1 and 2.

On evaluating the chromatograms it was established that in each case 4, well separated ninhydrin positive peaks were formed. The peaks represent: the initial amino acid, the alanyl utilized as active ester, the L-Ala-L-AA dipeptide and the L-Ala-D-AA dipeptide. The amino acids and diastereomeric dipeptides can be sorted out on the basis of the position of their peaks. Those amino acids which do not disturb one another's separation belong to the same group and can be determined in a single step. The groups are as follows: Asp, Ser, Pro, Ala-Thr, Glu, Ala-Ala, Val, Ile-Ala, Met, Leu. Fig. 3 shows the separation of the diastereomeric dipeptides obtained from the amino acids Thr, Glu and Ala. As it can be seen, separation is satisfactory, peaks can be evaluated, thus, quantitative determination is in no way hindered.

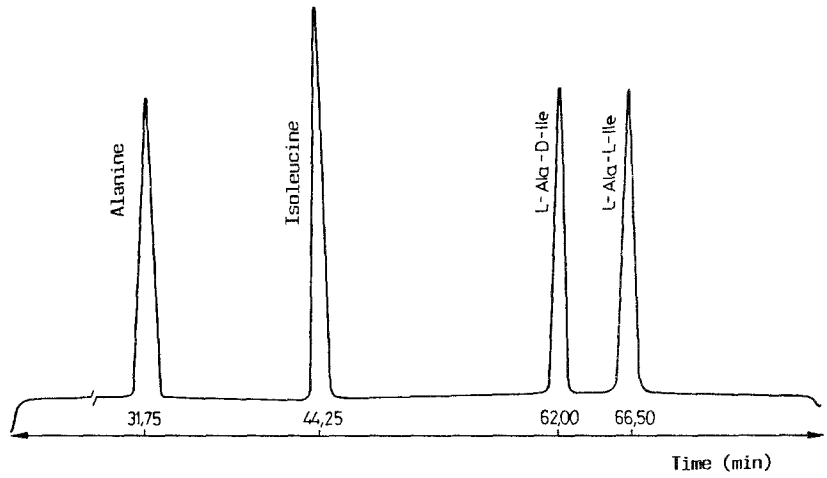

Fig. 2. Determination of D- and L-isoleucine

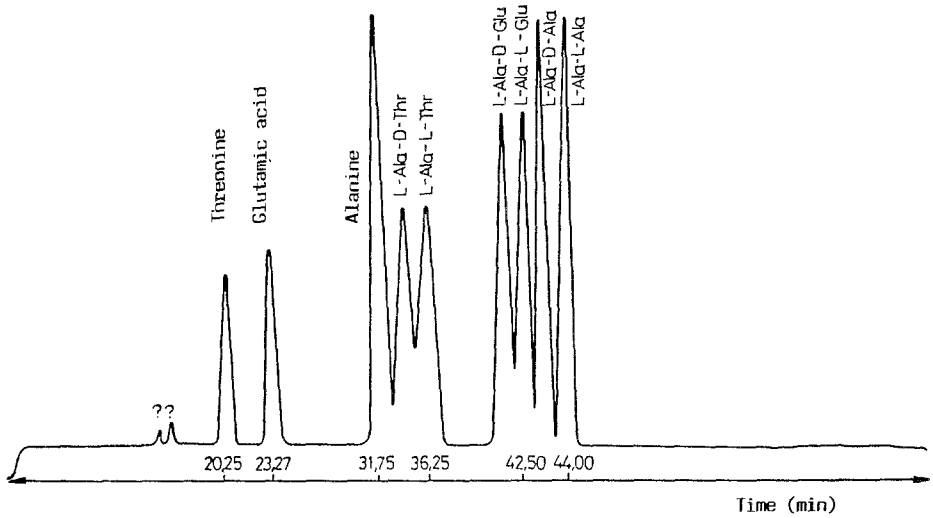

Fig. 3. Simultaneous determination of D- and L-threonine, glutamic acid and alanine

Accuracy of the determination

After finishing the development of the method, the D- and L-amino acid composition of synthesized amino acid mixtures was determined. Results are summarized in Table 1. Similarly to those presented in the table, analysis of every amino acid was carried out in 5 parallels in each concentration, however, only a few of them are discussed. As it can be seen in the table, the coefficient of variation decreases with increasing concentration, thus at higher concentrations the accuracy of determination is higher. However, even at the lowest concentration the value of the coefficient of variation does not reach 10, thus the method is reliable, its reproducibility is satisfactory. By appropriate grouping of the amino acids it becomes possible to determine the D- and L-isomers of several

Table 1. Determination of the D- and L-amino acids in various mixtures in the form of alanyl diastereomeric dipeptides

Material tested	Theoretical value (%)		Value measured (%)		Number of measure-ments	Standard deviation		Coefficient of variation	
	D	L	D	L		D	L	D	L
Glutamic acid	50	50	48.2	51.8	5	1.59	1.61	3.30	3.11
	25	75	26.4	73.1	5	0.97	2.11	3.67	2.89
	5	95	5.8	94.3	5	0.21	2.62	3.62	3.84
	1	99	0.95	98.7	5	0.043	2.74	4.53	2.78
Alanine	50	50	51.0	49.3	5	1.62	1.58	3.18	3.21
	25	75	25.2	75.1	5	1.05	2.05	4.17	2.73
	5	95	4.6	94.3	5	0.24	2.74	5.22	2.91
	1	99	1.10	98.8	5	0.055	2.90	5.00	2.94
Valine	50	50	51.1	49.7	5	1.74	1.63	3.41	3.28
	25	75	23.9	76.1	5	1.08	2.14	4.52	2.81
	5	95	5.1	93.9	5	0.26	2.81	5.10	2.99
	1	99	1.06	99.1	5	0.062	2.91	5.85	2.94
Isoleucine	50	50	50.1	51.1	5	1.69	1.72	3.37	3.37
	25	25	25.6	74.8	5	0.99	2.23	3.87	2.98
	5	95	4.9	94.6	5	0.32	2.62	6.53	2.77
	1	99	0.98	98.9	5	0.051	2.84	5.20	2.87

(two or three) amino acids in a single step. The method is suitable for the detection of at least 1% D-(or L-) amino acid in the presence of 99% L-(or D-) amino acid. The use of the method is suggested to all the laboratories which are in the possession of an amino acid analyzer and want to determine the D- and L-amino acids in synthetic amino acids, peptides or natural substances.

Conclusion

In spite of the fact that high performance liquid chromatographic methods have gained in popularity in the isolation and determination of optically active compounds, our experimental results have proven that laboratories (about 30 laboratories are carrying out feed analysis) having an amino acid analyzer, can also perform these analyses satisfactorily. An ion exchange chromatographic method for the quantitative determination of D- and L-amino acids has been developed in our laboratory.

The financial help provided by the Protein Program Bureau of the National Technical Development Committee, Budapest, is highly appreciated.

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Authors' address: J. Csapó, Faculty of Animal Science Kaposvár, Pannon Agricultural University, Dénesmajor 2, H-7401 Kaposvár, Hungary.