*Journal of Chromatography*, 135 (1977) 502–507


CHROM. 9921

## Note

# Determination of lysinoalanine with an automatic amino-acid analyzer

P. SLUMP

*Central Institute for Nutrition and Food Research TNO, Zeist (The Netherlands)*

(First received October 12th, 1976; revised manuscript received December 13th, 1976)

Recently, feeding studies were carried out[1] with $N^{\varepsilon}$-(DL-2-amino-2-carboxyethyl)-L-lysine (trivial name lysinoalanine, LAL), either as the free amino acid or as a residue within the structure of proteins or oligopeptides, obtained after treatment of proteins with alkali. In those studies, a reliable method was required for the determination of LAL in test samples and in blood and urine from test animals. Most methods of determination with automatic amino-acid analyzers described earlier were based on standard programmes for the separation of natural basic amino acids[2–4]. However, with such programmes LAL is not completely separated from other minor compounds that are often found in protein hydrolyzates and in physiological samples.

In this paper, two alternative chromatographic procedures are described by which this problem is at least partly solved.

## MATERIALS AND METHODS

### *Amino acids*

LAL was synthesized in this Institute[5]. Two diastereoisomers of LAL, $N^{\varepsilon}$-(L-2-amino-2-carboxyethyl)-L-lysine and $N^{\varepsilon}$-(D-2-amino-2-carboxyethyl)-L-lysine (abbreviated to L-L-LAL and L-D-LAL), were obtained in a pure form by Tas and Kleipool[6]. Ornithinoalanine [$N^{\delta}$-(DL-2-amino-2-carboxyethyl)-L-ornithine] and lysinomethylalanine [$N^{\varepsilon}$-(DL-1-methyl-2-amino-2-carboxyethyl)-L-lysine] were synthesized by the same procedure as used for the synthesis of LAL. S-$\beta$-(4-Pyridylethyl)-L-cysteine (PEC) was synthesized as described by Cavins and Friedman[7]. Other amino acids were commercial samples: $AA_5$ standard amino acid solution (Calbiochem, Los Angeles, Calif., U.S.A.), lanthionine, DL- and meso-forms (Nutr. Bioch. Corp, Cleveland, Ohio, U.S.A.), mixed DL- and DL-*allo*-$\delta$-hydroxylysine hydrochloride (Sigma, St. Louis, Mo., U.S.A.), L-ornithine monohydrochloride (Fluka, Buchs, Switzerland), L-1-methylhistidine monohydrate and L-3-methylhistidine (Calbiochem), DL-2,3-diaminopropionic acid hydrochloride (Fluka), tryptophan (Merck, Darmstadt, G.F.R.), cysteic acid (Koch-Light, Colnbrook, Great Britain), DL-$\gamma$-aminobutyric acid (Hoffman-La Roche, Basle, Switzerland), D-galactosamine hydrochloride (Baker, Phillipsburgh, N.Y., U.S.A.) and D-glucosamine hydrochloride (Biochemicals Res. Corp., Los Angeles, Calif., U.S.A.) and $N^{\varepsilon}$-methyl-L-lysine hydrochloride (Sigma). Methylarginines were not tested.

*Amino-acid analyzers*

Single column programmes were carried out on a Beckman Multichrom B liquid column chromatograph. The special LAL programme was carried out on an amino-acid analyzer built in this Institute[8]. However, other types of automatic amino-acid analyzers can be used without difficulty. Both analyzers were provided with an automatic sample injector and were connected with a Varian-CDS computer system.

*Chromatographic methods*

*(A) Special LAL programme.* The column used was packed with Bio-Rad Aminex A-4 spherical resin, height 50 cm, I.D. 0.9 cm, temperature 57°, elution rate 60 ml/h. Sodium citrate buffer (0.61 *N* sodium, 0.2 *M* citrate, pH 4.50) containing 59.6 g of sodium citrate dihydrate, 19.7 ml of concentrated hydrochloric acid, 5.4 ml of 17% Brij 35 solution and 0.1 ml of *n*-caprylic acid per litre was used.

*(B) Adapted 3-h single column programme for protein hydrolyzates.* A Beckman Type M 82 spherical ion-exchange resin column, height 50 cm, I.D./0.9 cm, was used at a temperature of 56° and an elution rate of 100 ml/h. Table I gives the stepwise buffer change programme and compositions of the buffers used for single-column operation.

TABLE I

BUFFER COMPOSITIONS AND BUFFER CHANGE PROGRAMMES USED FOR AMINO-ACID ANALYSIS

| *Parameter* | *pH* | | | | |
|---|---|---|---|---|---|
| | *3.45 ± 0.01* | *4.25 ± 0.02* | *6.28 ± 0.02* | *6.28 ± 0.02* | *>13* |
| Sodium ions (*N*) | 0.2 | 0.2 | 1.03 | 1.42 | 0.2 |
| Sodium citrate dihydrate (g) | 196.1 | 196.1 | 619.6 | 619.6 | — |
| Sodium chloride (g) | — | — | 213.2 | 462.4 | — |
| Sodium hydroxide (g) | — | — | — | — | 80 |
| Concentrated HCl (ml) | 116.6 | 83.7 | 6.3 | 6.3 | — |
| 17% aq. solution of Brij 35 (ml) | 54 | 54 | 54 | 54 | — |
| Phenol (g) | — | 10 | 10 | 10 | — |
| *n*-Caprylic acid (ml) | 1 | — | — | — | — |
| Final volume (l) | 10 | 10 | 10 | 10 | 10 |
| *Time schedule for buffers:* Minutes after starting the analysis | 0–33 and 170–190 | 33–78 | 78–114 | 114–160 | 160–170 |

RESULTS AND DISCUSSION

The separation of several amino acids on the Aminex-A-4 column with programme A is illustrated in Fig. 1. Hydroxylysine (Hyl) and galactosamine (GalN), which were not completely separated from LAL with the method used in earlier investigations[4], are well separated with this programme.

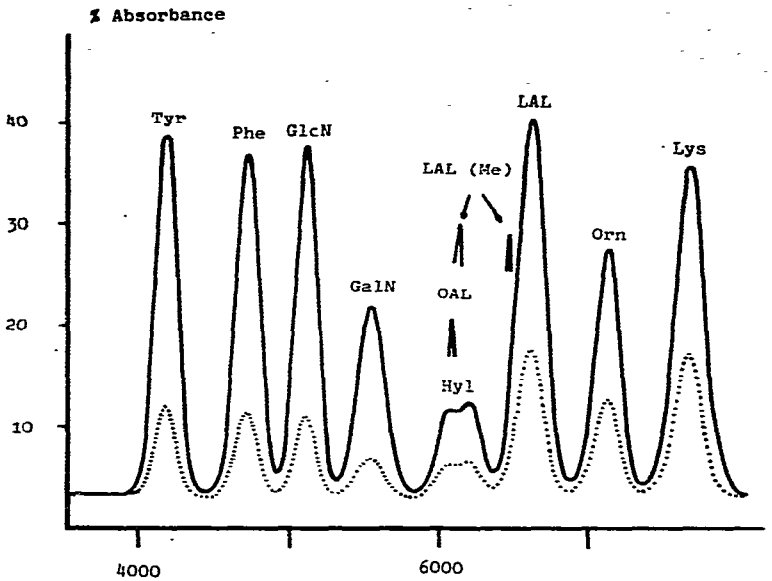


Fig. 1. Chromatography of LAL in the presence of some other amino acids. Column (50 × 0.9 cm): Aminex A-4 resin. Buffer: 0.61 *N* $Na^+$, pH 4.5. Solid line, absorbance at 570 nm; broken line, absorbance at 440 nm.

Some difficulties may arise with one of the isomers of lysinomethylalanine [LAL(Me)], an amino acid which theoretically can be formed during alkali treatment of proteins from threonine and lysine residues, as is shown in Fig. 1. Another isomer of LAL(Me) and ornithinoalanine (OAL) has the same retention time as one of the isomers of Hyl. With the method used by Ziegler *et al.*[3], it appeared that both isomers of LAL(Me) have the same retention time as OAL. L-L-LAL and L-D-LAL are eluted as a single peak in all of the chromatographic procedures tested. 2,3-Diaminopropionic acid [Ala(3$NH_2$)], found in alkali-treated wool[9], tryptophan, which interferes with LAL in the previous method[2,4], and other natural basic amino acids, including 1-methylhistidine [His($\pi$Me)], 3-methylhistidine [His($\tau$Me)] and $N^{\varepsilon}$-methyllysine [Lys(Me)], have retention times longer than that of lysine and are removed from the column in the regeneration step.

The results with chromatographic system B are illustrated in Fig. 2 for a mixture of common amino acids plus cysteic acid [Cys($O_3$H)], methionine sulphone [Met($O_2$)], $\gamma$-aminobutyric acid (NHB), GalN, GlcN, LAL, Hyl, ornithine and PEC. The positions of other amino acids, not present in the test sample, are also indicated in Fig. 2.

With system B, the LAL(Me) isomers give one peak with the same retention time as OAL, well separated from LAL. The isomers of lanthionine (Lan), also found in alkali-treated proteins[10], have the same retention time as serine and glutamic acid. The oxidized forms of Lan, obtained after oxidation with performic acid, are eluted at the same position as cysteic acid.

The lowest amount of LAL that could be detected is 0.2 $\mu$g, provided that a cuvette with a 10-mm optical path length is used. The maximal amount of hydrolyzed

TABLE II

COMPUTER REPORT CORRESPONDING WITH THE CHROMATOGRAM IN FIG. 2

Single column 4 buffer system.

| *Peak No.* | *Name* | *Type**** | *Comp St*§ | *Time (sec)* | *Corrected time (sec)* | *Area*** *(%)* | *Factor* |
|---|---|---|---|---|---|---|---|
| 1 | Cys($O_3$H) | P | 6.0596 | 874 | 874 | 2.1265 | 3602 |
| 2 | Asp | P | 13.3004 | 1605 | 1605* | 3.9034 | 4307 |
| 3 | Met($O_2$) | P | 7.5793 | 1728 | 1728 | 2.0533 | 4666 |
| 4 | Thr | P | 11.8998 | 1956 | 1956 | 4.0240 | 3738 |
| 5 | Ser | P | 10.5011 | 1946 | 1946 | 4.0867 | 3248 |
| 6 | Glu | P | 14.6996 | 2062 | 2062 | 3.8847 | 4783 |
| 7 | Pro 1/10§§ | P | 1.0541 | 2410 | 2410 | 0.2146 | 6208 |
| 8 | Cys | P | 12.0002 | 2675 | 2675 | 2.0827 | 7283 |
| 9 | Gly | P | 7.5002 | 2834 | 2834 | 4.2917 | 2209 |
| 10 | Ala | PB | 8.8986 | 2982 | 2982 | 3.9343 | 2859 |
| 11 | Val | | 11.6996 | 3246 | 3246* | 4.1906 | 3529 |
| 12 | Met | | 14.8989 | 3454 | 3454 | 3.9243 | 4799 |
| 13 | Ile | P | 13.1006 | 3648 | 3648 | 4.1193 | 4020 |
| 14 | Leu | P | 13.1002 | 3784 | 3784 | 4.2634 | 3884 |
| 15 | Nle | PB | 16.0016 | 3939 | 3939 | 5.2076 | 3884* |
| 16 | Tyr | P | 18.0999 | 4492 | 4492 | 4.1628 | 5496 |
| 17 | Phe | PB | 16.4984 | 4772 | 4772* | 4.1361 | 5042 |
| 18 | Nhb | | 8.9813 | 6004 | 6004 | 3.7943 | 2992 |
| 19 | GlcN | | 16.7986 | 6113 | 6113 | 3.6459 | 5824 |
| 20 | GalN | P | 10.3091 | 6275 | 6275 | 2.3265 | 5601 |
| 21 | Lal | PB | 11.2410 | 6414 | 6414 | 2.7768 | 5117 |
| 22 | His | P | 15.4990 | 6640 | 6640 | 4.5182 | 4336 |
| 23 | Hyl | P | 6.1998 | 6835 | 6835 | 1.4321 | 5472 |
| 24 | Orn | P | 7.9606 | 7204 | 7204 | 3.0669 | 3281 |
| 25 | Lys | P | 14.6006 | 7393 | 7393* | 5.0302 | 3669 |
| 26 | Pec | P | 24.0007 | 7955 | 7955 | 3.6688 | 8269 |
| 27 | Amm | | 1.7021 | 8270 | 8270* | 4.8680 | 442 |
| 28 | Arg | | 17.3998 | 9734 | 9734 | 4.2657 | 5156 |
| | | | 331.5845 | | | | |

* Reference peaks.

** Total area = 791,120.

*** P = no baseline just before and after peak; PB = baseline only after peak.

§ Comp St = total area × area (%) × Factor × $10^{-9}$ mg/l.

§§ Comp proline has to be multiplied with 10.

protein with which the columns can be loaded without causing clogging in the reaction coil is about 10 mg of protein. It therefore appears that the formation of LAL in proteins during alkali treatment or heating can be detected at levels higher than 20 ppm of the protein. The differences between duplicate analyses are 10–50% of the mean for LAL levels lower than 100 ppm and about 10% of the mean at levels higher than 100 ppm.

From our results and those of Sternberg *et al.*[11], it appears that hydrolysis for 22–24 h with 6 *N* hydrochloric acid at 108–110° is sufficient to liberate LAL completely from most proteins.

In serum and urine, LAL levels below 0.2 μg/ml are not detectable because of interference from small peptides and other ninhydrin-positive compounds.

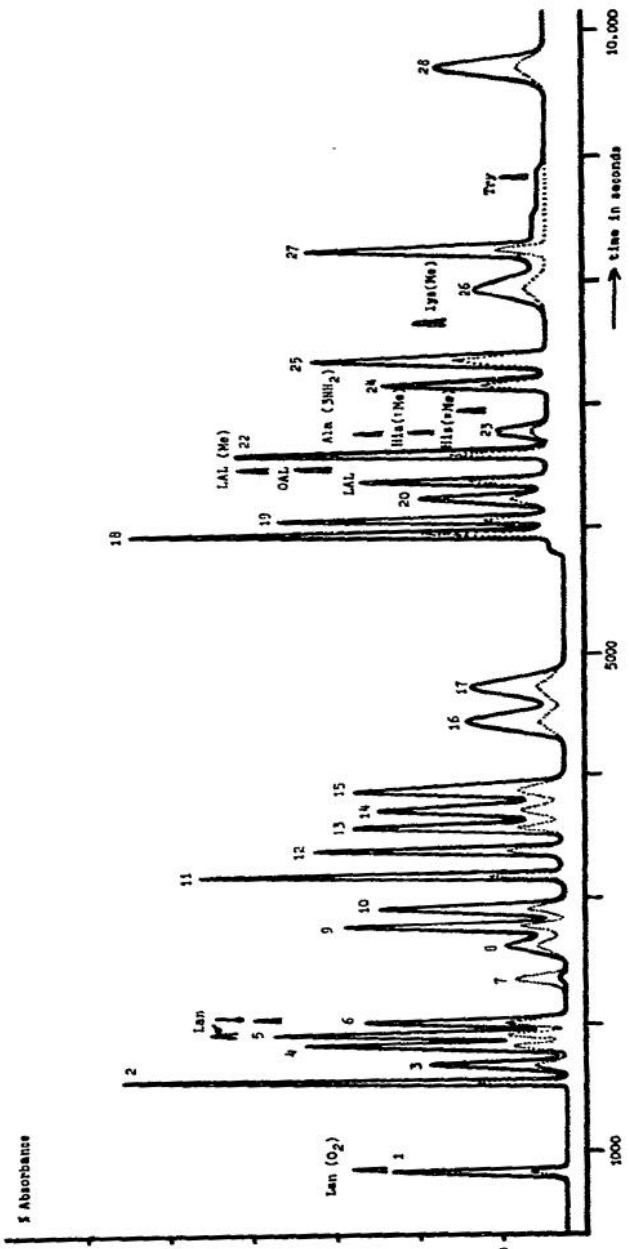


Fig. 2. Chromatography of a mixture of amino acids. Column (50 × 0.9 cm): Beckman Type M 82 resin. Buffers: see Table I. Peaks: see Table II. Solid line, absorbance at 570 nm; broken line absorbance at 440 nm.

Some whipping agents contain a large amount of LAL[12]. In these products, LAL is present in a bound form (poly- and oligopeptides) and in a free form. The determination of free LAL in such samples with the proposed methods was possible only after removal of most peptides. This removal was effected by precipitation with 10% trichloroacetic acid and filtration over polyacrylamide columns with Bio-Gel $P_2$.

## REFERENCES

1 A. P. de Groot, P. Slump, V. J. Feron and L. van Beek, *J. Nutr.*, 106 (1976) 1527.
2 Z. Bohak, *J. Biol. Chem.*, 239 (1964) 2878.
3 K. Ziegler, I. Melchert and C. Lürken, *Nature (London)*, 214 (1967) 404.
4 A. P. de Groot and P. Slump, *J. Nutr.*, 98 (1969) 45.
5 T. Okudo and H. Zahn, *Chem. Ber.*, 98 (1965) 1164.
6 A. C. Tas and R. J. C. Kleipool, *Lebensm. Wiss. Technol.*, 9 (1976) 360.
7 J. F. Cavins and M. Friedman, *Anal. Biochem.*, 35 (1970) 489.
8 P. Slump and A. M. Verbeek, *J. Chromatogr.*, 34 (1968) 401.
9 R. S. Asquith and J. J. Garcia-Dominquez, *J. Soc. Dyers Colour.*, 84 (1968) 155.
10 M. J. Horn, D. B. Jones and S. J. Ringel, *J. Biol. Chem.*, 138 (1941) 141.
11 M. M. Sternberg, C. Y. Kim and R. A. Plunkett, *J. Food Sci.*, 40 (1975) 1168.
12 M. Sternberg, C. Y. Kim and F. J. Schwende, *Science*, 190 (1975) 992.