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GAS–LIQUID CHROMATOGRAPHY OF FREE AMINO ACIDS IN THE HYALOPLASM OF RAT CEREBRAL, CEREBELLAR AND OCULAR TISSUES, AND IN SKELETAL AND HEART MUSCLE

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SUMMARY

The paper deals with the composition of amino acids in the hyaloplasm of cerebral tissue, cerebellum, eyeball, heart muscle and skeletal muscles. The investigations performed showed that: the most numerous groups of peaks were obtained from heart muscle (45), cerebellar tissue (43), skeletal muscle (36), eyeball (29) and cerebral tissue (25); and the highest molar levels corresponded to those of tryptophan in skeletal muscle, heart and cerebellum, proline in the heart, valine in the eyeball, and aspartic acid in the brain. Weight ratios indicated high contents of histidine, tyrosine and phenylalanine in the tissues of the skeletal muscles, the heart and cerebellum.

INTRODUCTION

The present work extends our investigations of the quantitative and qualitative composition of free amino acids contained in the hyaloplasm of different tissues and organs. In our previous work we determined the content of amino acids in the hyaloplasm from the liver and kidneys. This present paper deals with the results obtained from the following organs: frontal lobes of the brain, cerebellum, eyeball without muscles, heart muscle and skeletal muscles.

The analysis of free amino acids by gas—liquid chromatography (GLC) was performed according to the methods of Gehrke et al. [1, 2] and of Kaiser et al. [3]. Preparation of the hyaloplasm was performed as described by Chauveau et al. [4].

MATERIALS AND METHODS

Animals

Ten Wistar strain male rats, aged 3 months, each weighing 180 ± 30 g, were

used. Laparotomy and general perfusion were performed under urethane anesthesia, and perfusion fluid was introduced into the left ventricle. An isotonic solution of 0.75 *M* saccharose and Trition X-100 (0.5% at 4°) was used for perfusion (250 ml per animal). The tissues obtained were homogenized with the perfusion fluid at a ratio of 1:5.

Apparatus

The following equipment was used: Glass homogenizer (25 ml capacity) with a PTFE piston; K-24 and VAC 601 centrifuges (Janetzki Heinz K.-G., G.D.R.); ion-exchange columns were 12 mm × 150 mm; lyophilizing apparatus produced by VEB MLW Labortechnik, (G.D.R.); reaction vessels of our own design equipped with PTFE-lined screw-caps; automatic ultrasonic disintegrator UD-11 produced by Techpan; oil baths with a thermoregulator; evaporator produced by Büchi (Switzerland); Varian 3700 gas chromatograph with data analyzer CDS 111 C and an A 25 recorder; Hamilton 10-μl syringes; automatic pipettes (1000 μl and 100 μl) were produced by Eppendorf, G.F.R.

Reagents

Pure NaCl, 7 *N* ammonia solution and Dowex 50W-X8 100—200 mesh (H^+) were produced by Fluka, Buchs, Switzerland. Pure picric acid and gaseous HCl were also products of Fluka. *n*-Butanol Seq. grade and trifluoroacetic anhydride were from Pierce, Rockford, Ill., U.S.A. Standard amino acids were obtained from BDH (Poole, Great Britain), Mann Labs. (New York, N.Y., U.S.A.) and E. Merck (Darmstadt, G.F.R.). A calibration mixture was prepared by adding the successively estimated amino acids. 3% OV-17 on Varaport 30 100—200 mesh was from Varian, Palo Alto, Calif., U.S.A.

Preparation of amino acids

Homogenization. The tissues were homogenized at the ratio of 1:5.

Centrifugation and ultracentrifugation. The investigations were performed at ±4° according to the method of Chauveau et al. [4]. The homogenate was centrifuged at 600 *g* for 15 min, then the supernatant was decanted and centrifuged at 105,000 *g* for 60 min. Cell hyaloplasm was obtained in the supernatant, and 1% picric acid was added at a ratio of 1:5. The solution was centrifuged at 3500 *g* for 15 min, and the proteins were separated in the sediment. The deproteinized supernatant containing an excess of picric acid was introduced into the ion-exchange column.

Ion-exchange. In the following ion-exchange procedure a twenty-fold or greater excess of resin capacity to exchangeable ions placed on the column was maintained; i.e. 6 g of Dowex 50W-X8 100—200 mesh to 25 ml of supernatant. Six grams of ion exchanger placed in a 500-ml vial and covered with 7 *N* NH_4OH were mixed for 60 min. After sedimentation the fluid was decanted. The procedure was repeated two times. The column was then washed with bidistilled water to a pH of 7.5. The ion exchanger was regenerated with 3 *N* HCl (three times), and washed with water until a pH of 6.2 was obtained. Columns (15 mm × 150 mm) were filled half full with the wet resin. The supernatant with the picric acid was passed through the column at a rate of 2 ml/min. The surface was washed with water until the eluate was decolorised.

TABLE I

PERCENTAGE CONTENT OF PEAKS FROM THE SEPARATION OF AMINO ACIDS IN TISSUE HYALOPLASM

$$\text{Percentage content of peaks} = \frac{\text{Area of peak}}{\Sigma \text{ areas of all peaks}} \cdot 100.$$

No.	Percentage content of peaks				
	Brain	Cerebellum	Eyes	Muscle	Heart
1	—	0.10	—	—	0.43
2	2.74	1.51	5.34	3.39	1.42
3	2.65	2.22	5.40	1.63	1.16
4	—	0.34	—	—	0.69
5	11.42	7.27	9.02	4.68	2.21
6	—	1.25	—	—	0.70
7	1.86	1.14	4.51	1.40	0.85
8	—	0.86	0.39	0.17	1.27
9	0.65	1.42	1.64	0.55	1.77
10	—	4.57	0.53	20.49	5.33
11	3.44	1.87	5.94	1.81	1.40
12	—	0.62	0.59	1.87	1.34
13	—	0.24	—	1.38	0.35
14	0.08	0.19	0.14	0.34	0.16
15	—	0.10	—	—	0.19
16	0.14	1.43	—	0.30	2.53
17	—	0.12	—	—	0.25
18	—	—	—	—	0.49
19	20.82	13.73	2.42	0.38	0.32
20	—	0.24	—	—	0.35
21	1.86	1.25	3.37	0.94	0.56
22	—	—	—	—	0.22
23	—	0.20	—	0.25	0.52
24	0.41	0.72	1.25	1.03	0.58
25	—	0.23	—	—	0.35
26	1.57	1.51	—	1.10	1.36
27	16.32	1.70	2.19	0.99	1.65
28	1.80	1.26	2.35	0.78	0.90
29	—	0.32	—	0.25	0.42
30	1.22	1.02	2.38	0.72	0.89
31	0.17	0.76	0.75	0.64	0.75
32	28.93	14.13	30.60	6.07	13.13
33	0.05	0.62	0.36	0.53	0.97
34	0.07	1.31	1.06	1.13	1.84
35	0.06	0.81	0.62	1.17	1.30
36	—	1.39	—	0.81	1.07
37	—	2.09	2.15	1.75	1.38
38	0.24	1.19	1.59	1.16	1.91
39	0.13	2.45	1.06	1.37	2.79
40	1.57	7.89	3.87	18.72	13.69
41	0.46	1.16	0.69	1.26	2.33
42	1.28	13.92	4.94	23.51	21.26
43	—	2.34	1.80	3.24	2.25
44	—	1.90	0.94	1.84	2.64
45	—	2.97	—	1.93	5.04

Then 25 ml of 7 *N* NH_4OH and 30 ml of bidistilled and deionized water were passed through the column. The eluate and the washings were collected and mixed, and 55 ml of the mixture were lyophilized. The procedure was repeated according to the description of Zumwalt et al. [5].

Lyophilization. A 50-ml portion of the eluate and washings were collected in a vessel and quickly frozen in liquid nitrogen. The sample was placed in a 1000-ml condensor. After lyophilization 2 mg of dry sediment were attained and transferred to an esterification vessel.

Derivatization. The reaction was carried out according to the method of Gehrke et al. [2].

Packings and conditions of separation. Columns of Pyrex glass (200 cm × 6.35 mm O.D. × 2 mm I.D.) were packed with 3% OV-17 on Varaport 30 100—200 mesh, and 0.65% EGA on Chromosorb W AW 80—100 mesh. The temperature program was isothermal at 90° for 5 min, then increased at 7°/min up to 230° and then kept isothermal for 10 min. Injection temperature was 170°, flame ionization detector 250°; range 10^{-10}. Nitrogen flow-rate was 10 ml/min, hydrogen 30 ml/min, air 300 ml/min. Chart speed was 1 cm/min, with CDS 111 C computer read-out. Each sample of the amino acids obtained from particular organs and tissues was subjected to chromatographic analysis in triplicate; the total number of chromatograms was 150.

RESULTS AND DISCUSSION

The investigations were carried out for 70 days. During this time 50 final products of derivatized amino acids from the tissues examined were prepared. The products of the derivatization reaction were *n*-butyl N-trifluoroacetyl esters, yellow in colour, which were injected into the OV-17 phase column. Table I shows the number and percentage of peak areas obtained from the various tissues. The analysis, performed according to the method of Amico et al. [6], corresponded to a number of standard amino acids in our laboratory. Thus, it was possible to determine the following amino acids: alanine (2); threonine (3); glycine + serine (5); valine (7); leucine + isoleucine (11); proline (21); methionine (24); histidine (26); asparagine (27); phenylalanine (28); tyrosine (30); glutamic acid + lysine (32); and tryptophan (40). Table II lists identified amino acids according to Zumwalt et al. [7]. Pellizzari et al. [8] and Gehrke et al. [9]. Table I is arranged according to increasing retention time and relative molar response (RMR); it also provides information about the composition of the hyaloplasm of the investigated tissues. This list does not include identification of peak 19 which provided support for the presence of γ-aminobutyric acid, which was identified on the basis of the literature data [10], as well as numerous articles published in the Journal of Neurochemistry. In Table II special attention is paid to glycine, serine, glutamic acid, lysine, leucine, isoleucine and aspartic acid in brain tissue. From among the amino acids that we have in our laboratory, norvaline was chosen as the internal standard.

Table III shows a high weight of the following amino acids: histidine, tyrosine, phenylalanine, proline, aspartic acid and valine in heart muscle, and threonine, valine and methionine in the cerebellar tissue. Tables I—III and Figs. 1—5, showing the peaks, were the basis of our analysis and also helped us draw the final conclusions:

TABLE II

PERCENTAGE CONTENT OF AMINO ACIDS IN TISSUES ACCORDING TO RELATIVE MOLAR RESPONSE

$$\text{Percentage molar} = \frac{\text{area of amino acid}}{100 \cdot \text{RMR of glutamic amino acid}}$$

where area of amino acid = area of the amino acid peak from chromatogram, RMR = relative molar response, RMR of glutamic amino acid = $\frac{\text{Molar response of amino acid}}{\text{Molar response of glutamic acid}}$

Amino acid	Percentage molar				
	Brain	Cerebellum	Eyes	Heart	Muscle
Ala	3.0 ± 0.00	2.4 ± 0.6	5.4 ± 0.6	3.0 ± 2.0	7.2 ± 2.8
Thr	3.0 ± 0.00	3.6 ± 1.4	5.8 ± 1.2	2.1 ± 0.9	3.0 ± 1.0
Gly + Ser	15.00 ± 0.00	13.6 ± 4.4	11 ± 1.6	5.0 ± 3.0	10.2 ± 1.8
Val	1.8 ± 0.2	2.2 ± 0.8	5.8 ± 0.2	1.3 ± 1.7	2.5 ± 1.5
Leu + Ile	4.2 ± 0.8	3.4 ± 1.6	6.4 ± 1.6	3.0 ± 1.0	3.3 ± 5.7
Pro	1.8 ± 0.2	1.8 ± 1.2	3.8 ± 0.2	0.7 ± 0.3	1.4 ± 0.6
Met	0.3 ± 0.00	1.1 ± 0.9	1.0 ± 0.00	1.2 ± 1.8	1.6 ± 1.4
His	1.0 ± 0.00	3.4 ± 1.6	2.0 ± 0.00	2.8 ± 1.2	1.7 ± 1.3
Asp	21.6 ± 1.4	3.0 ± 1.0	5.8 ± 10.2	3.4 ± 1.6	1.7 ± 0.3
Phe	1.8 ± 0.2	2.0 ± 0.00	2.6 ± 0.4	1.6 ± 0.4	1.2 ± 0.8
Tyr	1.0 ± 0.00	1.4 ± 0.6	4.6 ± 8.4	1.4 ± 1.6	1.0 ± 1.0
Glu + Lys	37.2 ± 1.8	35.6 ± 7.4	35.2 ± 3.8	32.3 ± 5.7	12.9 ± 1.1
Try	1.2 ± 0.8	17.1 ± 1.9	5.0 ± 1.0	31.1 ± 8.9	41.2 ± 4.8

TABLE III

ANALYSIS OF TISSUE AMINO ACIDS IN 2 mg OF LYOPHYLIZATE OF CELL CYTOSOL

Values were calculated from the formula $G_X = G_S \frac{A_X \cdot RMR_S \cdot M_X}{A_S \cdot RMR_X \cdot M_S}$

where G_X = grams of amino acids in a given number of specimens, A_X = peak area of the investigated amino acid, RMR_S = relative molar response of the internal standard, M_X = molecular weight of the estimated amino acid, A_S = peak area of the internal standard, RMR_X = relative molar response of the estimated compound, M_S = molecular weight of the standard, G_S = grams of internal standard.

Amino acid	Muscular tissue (μg)	Heart muscle (μg)	Cerebellar tissue (μg)
Ala	76.53 ± 0.31	80.01 ± 1.23	75.43 ± 4.11
Thr	101.69 ± 1.65	106.95 ± 3.72	108.34 ± 1.36
Val	99.07 ± 1.58	109.16 ± 9.54	104.13 ± 10.14
Pro	96.93 ± 3.91	120.81 ± 8.83	98.19 ± 15.99
Met	123.19 ± 7.53	155.67 ± 12.38	143.13 ± 9.40
His	188.06 ± 10.14	188.34 ± 8.39	172.59 ± 0.69
Asp	114.70 ± 4.48	120.04 ± 2.61	114.96 ± 3.88
Phe	142.02 ± 5.45	156.19 ± 15.40	148.82 ± 5.54
Tyr	154.43 ± 12.28	174.36 ± 9.32	161.83 ± 17.81

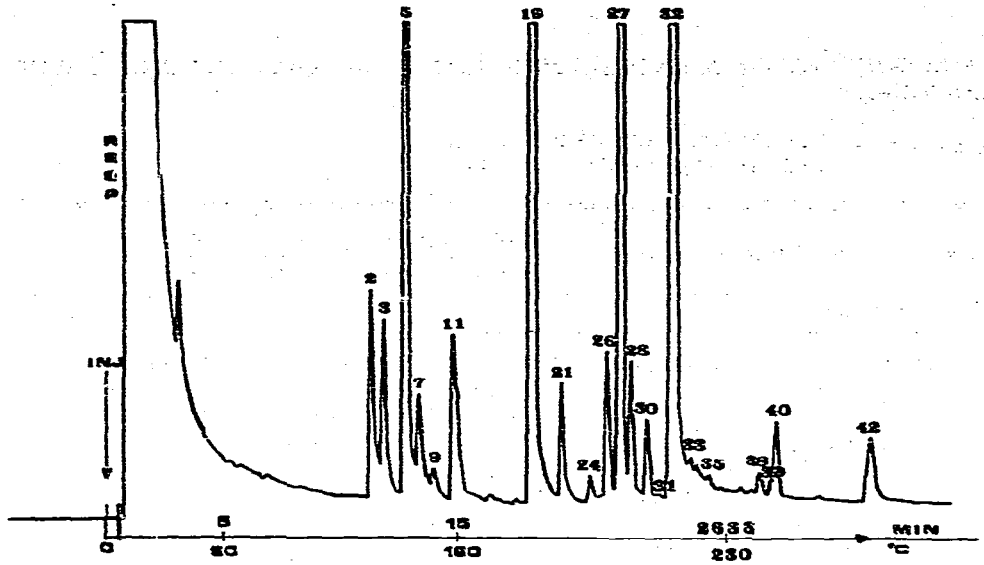

Fig. 1. Chromatogram of amino acids, cleaned by cation exchange, in the hyaloplasm of rat cerebral tissue. Column: 3% OV-17 on Varaport 30. Conditions: initial temperature 90°, isothermal at 90° for 5 min, and then 7°/min up to 230°, and isothermal for 10 min; range $10^{-10} \times 64$.

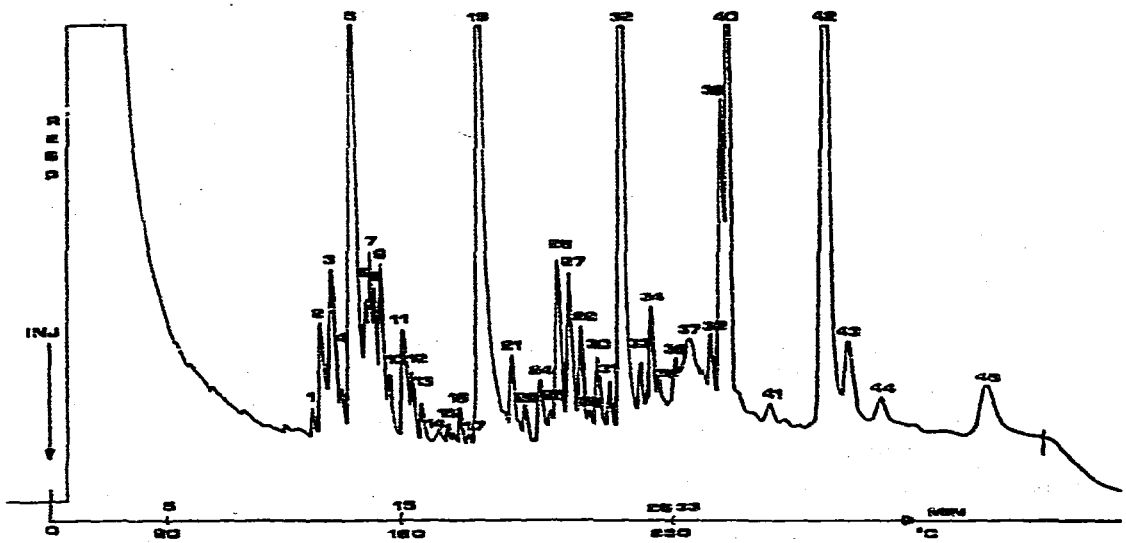

Fig. 2. Chromatogram of amino acids, cleaned by cation exchange, in the hyaloplasm of rat cerebellar tissue. Column and conditions as in Fig. 1.

(1) The method can be used in the investigation of free amino acids in cell hyaloplasm.

(2) A separation column with EGA phase and ornithine as the internal standard should be introduced.

(3) On the basis of the literature data and the available amino acids, a calibration mixture containing more standards should be prepared.

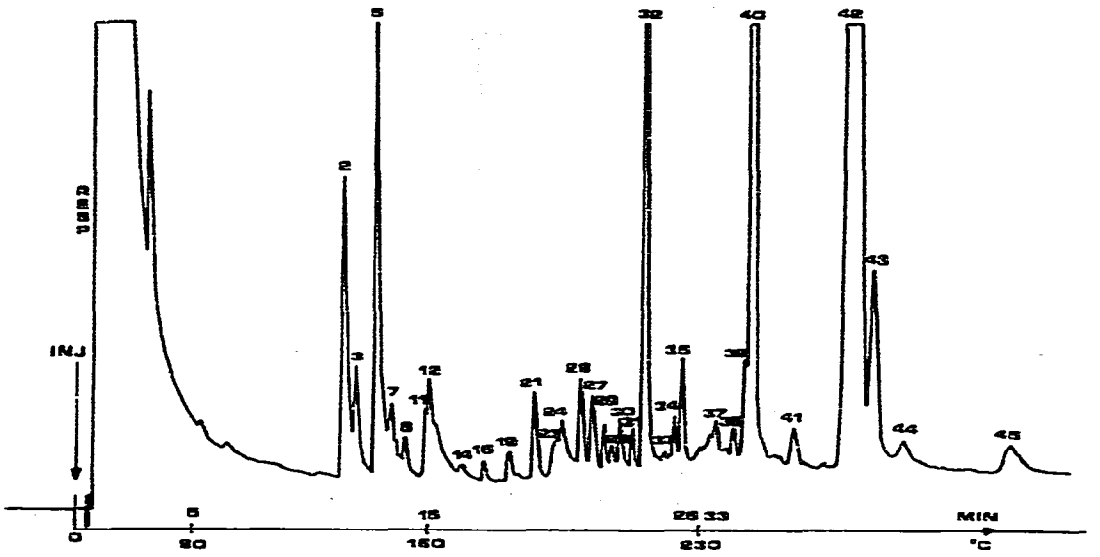

Fig. 5. Chromatogram of amino acids, cleaned by cation exchange, in the hyaloplasm of rat femoral tissue. Column and conditions as in Fig. 1.

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