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Note

Resolution of 52 ninhydrin-positive compounds with a high-speed amino acid analyser

Determination of carnosine and homocarnosine in biological materials

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Many cases of inborn errors of amino acid metabolism have been described [1]. Perry et al. [2] described carnosinaemia, a metabolic disorder associated with neurological disease and mental defects involving impaired cleavage of midazole dipeptides. Sjaastad et al. [3] found elevated concentrations of nomocarnosine in cerebrospinal fluid of family members having spastic paraplegia.

In such cases, identification of carnosine (β-alanylhistidine) and homocarnosine (γ-aminobutyrylhistidine) in physiological fluids was complicated. Perry et al. [4] described two-dimensional paper chromatographic and high-voltage electrophoretic methods, but they were time-consuming and only semi-quantitative. They also used an amino acid analyser to identify these peptides [4]. Carnosine and homocarnosine were eluted after 1,111 and 1,114 min, respectively. As each peak width was about 10 min, it was difficult to resolve the peaks completely as the difference in retention times between carnosine and homocarnosine was only 3 min.

Adriaens et al. [5] reported the identification of these peptides with an amino acid analyser using lithium buffers. The peak resolution of the peptides was about 50% and the retention times were over 21 h.

In this paper, we report the determination of carnosine and homocarnosine in physiological fluids with a high-speed amino acid analyser.

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EXPERIMENTAL

Materials

L-Carnosine was purchased from Tokyo Chemical Industry (Tokyo, Japan) and L-homocarnosine sulphate, δ-amino-*n*-valeric acid and D-glucosaminic acid from Nakarai Chemical (Kyoto, Japan). Titanium(III) chloride was purchased from Pierce (Rockford, IL, U.S.A.) and other reagents from Wako (Osaka, Japan) and Nakarai Chemical.

A standard solution of ninhydrin-positive compounds was prepared as described in a previous paper [6].

Amino acid analysis

A Hitachi 835 high-speed amino acid analyser equipped with an autosampler, data processer, graphic printer and recorder was used. The experimental conditions are summarized in Table I. The programs for changing the buffer and temperature were modified following the Hitachi instruction manual.

TABLE I

EXPERIMENTAL CONDITIONS FOR AMINO ACID ANALYSIS

Column: 250 × 2.6 mm I.D. stainless steel, packed with Hitachi Custom Ion-exchange resin 2619.

Flow-rates: pump 1 (buffer), 0.275 ml/min (190 kg/cm²)
pump 2 (ninhydrin), 0.300 ml/min (30 kg/cm²)

Programme:

Buffer change		Temperature change	
1 → 2	66 min	34 → 43°C	32 min
2 → 3	82 min	43 → 47°C	69 min
3 → 4	128 min	47 → 68°C	101 min
4 → 5	164 min	68 → 45°C	153 min
5 → 6	191 min	45 → 34°C	218 min
6 → 1	203 min		

Buffer compositions:

Buffer	Lithium concentration (*N*)	pH	LiOH (g/l)	Li citrate (g/l)	LiCl (g/l)	Citric acid (g/l)	Ethanol (ml/l)	Thio-diglycol (ml/l)	25% Brij-35 (ml/l)
1st	0.155	3.00	—	9.80	2.12	35	40	5	4
2nd	0.255	3.70	—	9.80	6.36	13	30	5	4
3rd	0.805	3.46	—	9.80	29.67	12	—	—	4
4th	1.000	4.40	—	9.80	38.14	3.3	—	—	4
5th	1.200	7.07	—	47.00	29.68	—	—	—	4
6th	0.200	—	8.40	—	—	—	—	—	4

Ninhydrin reagent:

Ninhydrin	80 g
Methylcellosolve	3 l
5 *M* sodium acetate buffer (pH 5.5)	1 l
Titanium(III) chloride (15%)	6.8 ml

All of the buffers were passed through a stainless-steel pre-column (120 × 4 mm I.D.) packed with Hitachi Custom Ion-exchange Resin 2650 to remove ammonia.

Sample preparation

Urine. Samples from a band-shaped keratopathy patient were used. A 10-ml volume of 24-h urine that had been preserved by adding toluene was deammoniated by the method of Benson and Patterson [7]. The residue was resuspended in and diluted to 10 ml with 0.01 *N* hydrochloric acid.

Cerebrospinal fluid (CSF). CSF was obtained by lumbar puncture from a patient with Reye's syndrome, collected in a centrifuge tube and after 10 mg/ml of sulphosalicylic acid had been added, was centrifuged at 10,000 *g* for 10 min. The deproteinized supernatant was analysed.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatograms of 52 ninhydrin-positive compounds obtained with the Hitachi 835 high-speed amino acid analyser. The total analysis time was 195 min.

Excellent resolution of these compounds was obtained, except for cystathionine—*allo*-isoleucine and 5-hydroxylysine—*allo*-hydroxylysine. δ-Amino-*n*-valeric acid (AVA) (144.68 min) and D-glucosaminic acid (13.94 min) were used as internal standards instead of norleucine, the peak of which overlapped the tyrosine peak. These internal standards were used because the sample size in the high-speed amino acid analyser was only 50 μl.

The colour yields of carnosine, asparagine and anserine were lower than in a standard Hitachi KLA-5 amino acid analyser. As the ninhydrin reaction time with the high-speed amino acid analyser was only 1.706 min, it was unavoidable that the peak areas at 570 nm were smaller than those at 440 nm, and we quantified these compounds at 440 nm, as for proline.

Carnosine and homocarnosine were eluted after 175.18 and 177.18 min, respectively, and the resolution was about 30% at 570 nm and 80% at 440 nm. The colour yield of homocarnosine was greater than that of carnosine at 570 nm, the peak-area ratios (440/570 nm) being 1.26 for carnosine and 0.18 for homocarnosine. These ratios were used for the identification of these peptides.

Fig. 2 shows a typical chromatogram of the amino acids in CSF. A large amount of glutamine was present, but the other amino acids were found in only very low concentrations. Homocarnosine was clearly identified by the retention time and the peak-area ratio, which agreed with those of the authentic compound.

Carnosine in the urine was also identified by the same method, as shown in Fig. 3. Some homocarnosine was present in this sample. This urine sample contained many ninhydrin-positive compounds that could not be identified clearly.

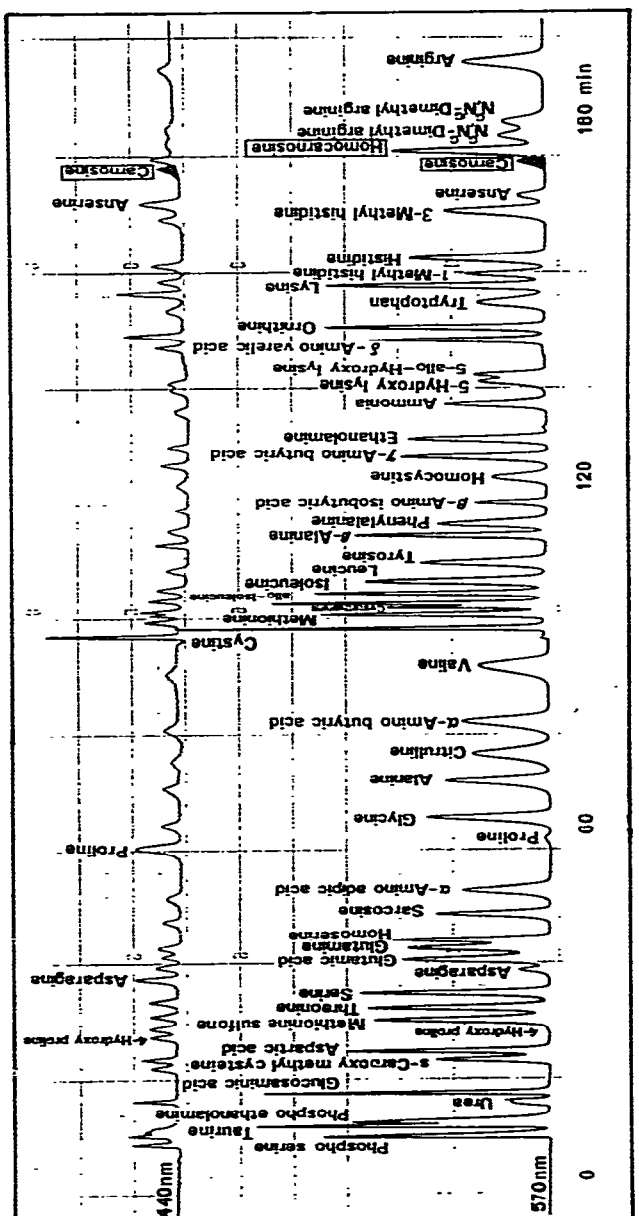

Fig. 1. Typical chromatogram of 52 ninhydrin-positive compounds obtained with a high-speed amino acid analyser. The amount of each amino acid to be analysed was 2.5 nmole. The following amino acids were prepared: homoserine (0.625 nmole); cystathionine (1.0 nmole); S-carboxymethylcysteine, N^G,N^G-dimethylarginine and N^G,N'^G-dimethylarginine (1.25 nmole); proline, sarcosine, β-alanine and anserine (5 nmole); and urea (250 nmole).

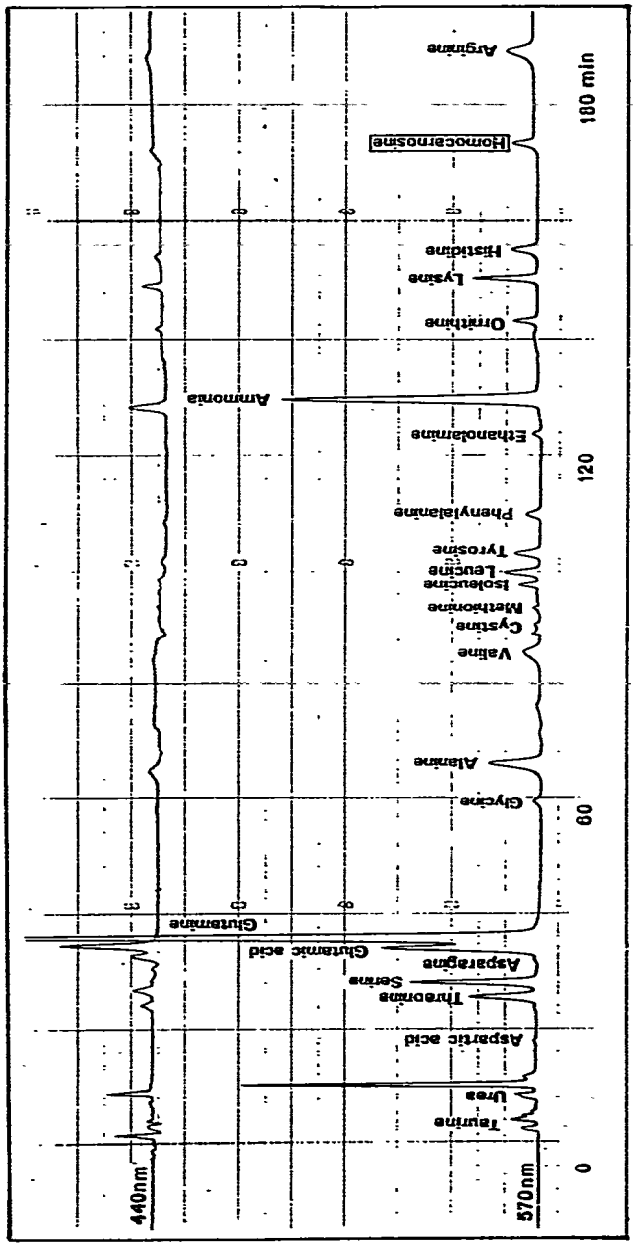

Fig. 2. Chromatogram of amino acids in 33.3 µl of CSF collected from a Reye's syndrome patient.

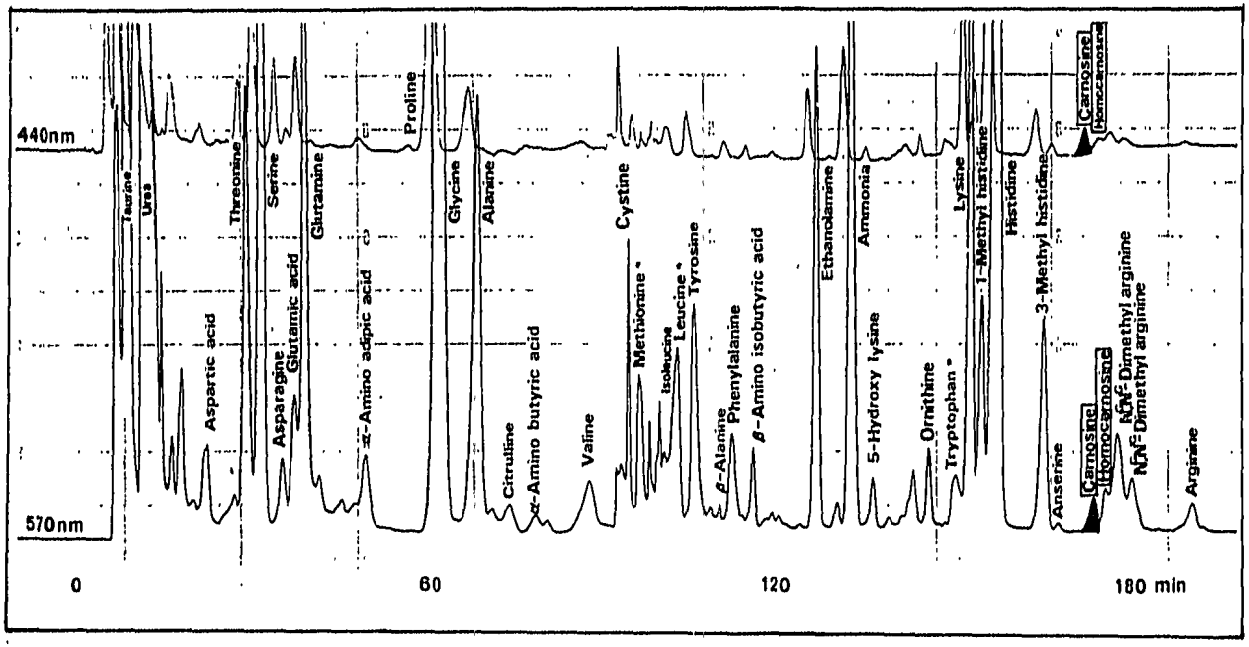

Fig. 3. Chromatogram of amino acids in 50 μl of urine collected from a band-shaped keratopathy patient. The asterisks indicate peaks that seemed to contain other unknown compounds.

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