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Quantification of physiological amino acids by gradient ion-exchange high-performance liquid chromatography

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ABSTRACT

A single-column gradient lithium ion-exchange chromatographic method with post-column derivatization and fluorimetric detection for the quantification of physiological amino acids is described. The method runs automatically, requires a minimum of sample preparation, separates all amino acids in plasma and cerebrospinal fluid, and most compounds in brain extract, in addition to some amino acids used therapeutically and in pharmacological studies. About 40 compounds can be quantitated within a run time of 3 h. The within-assay and between-assay coefficients of variations for principal amino acids in plasma samples are satisfactory. The system has performed conveniently and with high stability in the daily routine work and is cost-saving based on laboratory-prepared buffers.

INTRODUCTION

Determination of amino acids in physiological fluids, *e.g.* plasma and cerebrospinal fluid (CSF), originally involved separation by ion-exchange chromatography followed by post-column reaction with ninhydrin, giving derivatives that could be detected photometrically [1]. The sensitivity was only moderate and the quantitation of all physiological amino acids on a single column took 21 h [2], or 3 h for the large neutral amino acids alone [3], or required expensive apparatus that was dedicated to amino acid analysis.

The sensitivity was increased *ca.* ten-fold [4] after it was shown that amino acids and *o*-phthalaldehyde in the presence of mercaptoethanol produced a fluorescent adduct [5], and the reaction was applied to amino acid chromatography in post-column detection [6,7]. Furthermore, the introduction of high-performance liquid chromatography (HPLC) technology used in combination with a variety of pre-column derivatizations, a reversed-phase column, and fluorimetric [8–12] and/or electrochemical detection [13,14] considerably shortened the analysis time and added to the sensitivity.

One major disadvantage of the reversed-phase mode of amino acid analysis is the varying stability of the reaction products, which require precise control of reaction timing and chromatographic parameters for reliable quantitative application [15]. These variables could explain, in part, the greater coefficients of variation (C.V.) for the reversed-phase relative to the conventional method [8]. Another drawback is that physiological amino acids may be eluted together, or an amino acid may be co-eluted with the protein-precipitating agent.

In recent years, new resins in the form of robust, small particles of great homogeneity have been marketed, allowing amino acid analysis by ion-exchange HPLC with post-column derivatization. This technology was recently applied on a dedicated analyser involving the use of two temperatures and four [16] or three [17] lithium buffers. By the present method, all physiological

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and some additional amino acids are separated by a single-column, isothermal two-buffer system, which can run automatically for days with high accuracy, sensitivity and stability.

EXPERIMENTAL

Apparatus

The HPLC system (Kontron, Zurich, Switzerland) consisted of two Model 420 dual-piston pumps equipped with small pump heads (delivering from 10 μl/min to 2 ml/min), a Model 491 dynamic mixer with a 0.6-ml mixing chamber, a Model 830 column oven, a Model 460 automatic sample injector with programmable injection volume and a cooling option connected to a Savant RWC 825 constant temperature bath–recirculator giving a sample temperature of 4°C, a Model 800 printer–plotter, an Anacomp 220 HPLC computer and a SFM 23 LC spectrofluorometer with a 150-W xenon high-pressure lamp, a 20-μl flow-through cell and 10 nm spectral bandwidth for excitation and 20 nm for emission. The excitation/emission wavelengths were 340/448 nm (uncalibrated). The amino acids were separated on a 150 mm × 3 mm I.D. column with 5 μm particle size lithium cation-exchange material and equipped with a 20 mm × 3 mm I.D. guard column (Pickering Labs., Mountain View, CA, USA). The stationary phase in both the analytical and guard columns was a fully sulphonated copolymer of divinylbenzene and styrene.

Reagents

Water purified by reverse osmosis (Elgastat UHQ II, Bucks, UK) was used for the preparation of buffers and other solutions. The chemicals were of analytical grade or better. Titriplex III (ethylenediaminetetraacetic acid disodium salt dihydrate), sodium hydroxide, 2,2-thiodiethanol, hydrochloric acid fuming 37%, disodium tetraborate decahydrate, 5-sulphosalicylic acid and lithium hydroxide monohydrate were purchased from Merck (Darmstadt, Germany); trilithium citrate tetrahydrate and *o*-phthaldialdehyde from Fluka (Buchs, Switzerland); phenol liquefied 80% from BDH (Poole, UK); and 2-mercaptoethanol and the majority of the amino acid compounds from Sigma (St. Louis, MO, USA).

OPA reagent. To a solution of 250 ml of 0.2 *M* sodium borate buffer (pH 10), 25 ml of 1 *M* sodium hydroxide and 0.5 g of Titriplex III made up to 500 ml with water were added 450 mg of *o*-phthaldialdehyde in 5 ml of ethanol followed by 1 ml of 2-mercaptoethanol. The solution was filtered through a 0.45-μm Sartorius cellulose acetate filter (Göttingen, Germany) and stored in an amber bottle under nitrogen while in use. The rate of the OPA reagent supply to the column effluent was 0.42 ml/min.

Buffer A. The 0.24 *N* lithium citrate buffer (pH 2.27) was prepared by dissolving 89.3 g of trilithium citrate tetrahydrate, 125 ml of 2 *M* lithium hydroxide, 25 ml of thio-diethanol and 6.25 ml of phenol in 4.5 l of water. This solution was titrated with 6 *M* HCl to pH 2.27, made up to 5.0 l with water, re-adjusted to pH 2.27, and filtered through a 0.45-μm filter. During use, the buffer was stored in an amber bottle under helium.

Buffer B. The 0.64 *N* lithium citrate buffer (pH 7.50) was prepared and stored in the same way as buffer A, except that 5.0 l contained 277.3 g trilithium citrate tetrahydrate, 5.0 g of Titriplex III were added, and it was titrated to pH 7.50.

Collection of physiological samples

Blood was collected under light stasis from the antecubital vein of healthy subjects in the morning after an overnight fast. Samples were collected in EDTA-3K tubes (Vacutainer), giving a final concentration of 1 mg EDTA-3K per ml of blood. Plasma was separated by centrifugation at 2000 *g* for 15 min at 4°C and used immediately or stored at −80°C.

Samples of CSF were collected in the morning from neurological patients in recumbent position and stored at −80°C until analysis.

Rats were decapitated by guillotine and trunk blood was collected in EDTA-3K tubes and processed like human blood. A half brain cortex, *ca.* 250 mg, was rapidly dissected free and placed in a tared tube containing 1.50 ml of 0.4 *M* ice-cold perchloric acid. After weighing and homogenization, the brain extract was separated by

centrifugation at 50 000 *g* for 20 min at 4°C and used immediately or stored at −80°C. The solution was air-pressed through a 30 000 NMWL membrane filter (Novacell, Filtron Technology, Clinton, MA, USA) before analysis.

Standard and sample preparation

Stock solutions of amino acid standards (2.5 μmol/ml of each amino acid) were prepared in 0.1 *M* HCl and, after appropriate dilution in 0.1 *M* HCl, used for peak identification and determination of retention time and linearity of response.

A plasma standard, containing all detectable amino acids in plasma in the approximate proportions they appear in plasma (see Table II), was prepared in 0.1 *M* HCl and used for the determination of response factors relative to the internal standard, α-aminoadipic acid. A brain extract standard was prepared according to the same principles and procedures.

A 200-μl volume of human or rat plasma was mixed with 50 μl of a cooled solution containing 16% (w/v) sulphosalicylic acid and 400 nmol/ml α-aminoadipic acid. The tube was vortex-mixed, and after standing for 10 min at 4°C, the mixture was re-vortexed and centrifuged at 2000 *g* for 10 min at 4°C. The clear supernatant was air-pressed through a 30 000 NMWL membrane filter, and 7 μl of the filtrate (100 μl in total) were injected into the HPLC system.

A 200-μl volume of human CSF was mixed with 50 μl of a cooled solution containing 8% (w/v) sulphosalicylic acid and 100 nmol/ml α-aminoadipic acid. The tube was vortex-mixed and, after standing for 10 min at 4°C, the mixture was re-vortexed and air-pressed through a 30 000 NMWL membrane filter, and 20 μl were injected into the HPLC system.

Chromatographic procedures

The column was regenerated and equilibrated by pumping 0.3 *M* lithium hydroxide solution through it for 5 min at 0.3 ml/min followed by buffer A first for 13 min at 0.3 ml/min, then for 2 min at 0.2 ml/min. The gradient profile used for the separation of the amino acids is shown in Table I. The column temperature was maintained at 50°C, and the gradient was started immediately after the injection.

RESULTS AND DISCUSSION

A chromatogram of a standard solution containing 600 pmol each of 40 amino compounds is shown in Fig. 1. The mean retention times of amino acids in plasma and CSF are shown in Table II. The resolution is satisfactory and is sufficient to quantitate naturally occurring amino acids in physiological fluids, as well as some amino acids used therapeutically and in pharmacological studies, *e.g.* DOPA and 5-hydroxytryptophan.

TABLE I

GRADIENT PROFILE USED FOR SEPARATION OF AMINO ACIDS

Buffer A is 0.24 *N* lithium citrate buffer (pH 2.27), and buffer B is an 0.64 *N* lithium citrate buffer (pH 7.50).

Time (min)	Buffer A (%)	Buffer B (%)	Flow-rate (ml/min)	Gradient
0	100	0	0.2	Linear
35	95	5	0.2	Isocratic
40	95	5	0.2	Linear
75	85	15	0.2	Linear
80	40	60	0.3	Linear
110	35	65	0.3	Linear
145	0	100	0.3	Isocratic
195	0	100	0.3	

TABLE II

RETENTION TIMES (RT), COEFFICIENTS OF VARIATION (C.V.) AND CONCENTRATIONS IN PLASMA, CEREBROSPINAL FLUID (CSF) AND RAT BRAIN CORTEX OF INDIVIDUAL AMINO ACIDS

Results are presented as the mean ± S.D. (n = 8 for RT, within-run and between-run C.V., and n = 6 for plasma, CSF and brain cortex samples).

Amino acid	RT [a] (min)	Within-run C.V. (%)	Between-run C.V. (%)	Human plasma (nmol/ml)	Human CSF (nmol/ml)	Rat plasma (nmol/ml)	Rat brain cortex [b] (nmol/g wet weight)
Tau	4.34 ± 0.03	4.4	2.7	50 ± 13	11.1 ± 1.1	353 ± 27	8238 ± 363
Pea	5.93 ± 0.02	7.3	28.2	4.1 ± 0.9	6.1 ± 2.2	24 ± 4	2064 ± 87
Asp	25.6 ± 0.5	13.3	11.5	Trace	Trace	29 ± 4	2764 ± 179
Thr	33.4 ± 0.7	3.4	11.8	126 ± 28	37.8 ± 6.9	322 ± 55	481 ± 79
Ser	34.2 ± 0.8	6.1	14.0	99 ± 19	27.7 ± 5.1	347 ± 36	937 ± 130
Asn	36.7 ± 0.9	6.6	5.1	50 ± 11	6.9 ± 1.5	82 ± 6	108 ± 25
Glu	39.8 ± 0.9	6.2	11.7	31 ± 10	3.9 ± 0.6	131 ± 4	12 036 ± 583
Gln	42.3 ± 1.0	5.4	5.1	689 ± 176	735 ± 59	863 ± 70	8333 ± 438
Gly	56.4 ± 1.8	4.5	6.2	201 ± 54	9.0 ± 2.6	439 ± 28	1004 ± 130
Ala	58.8 ± 2.1	4.6	6.8	272 ± 34	35.8 ± 6.7	409 ± 27	764 ± 70
Cit	60.7 ± 2.4	4.5	11.3	36 ± 10	3.5 ± 0.9	90 ± 5	20 ± 3
Abu	62.2 ± 2.2	7.0	12.0	25 ± 9	4.9 ± 0.7	26 ± 6	8 ± 3
Val	64.8 ± 2.0	6.2	6.9	234 ± 25	19.4 ± 5.3	291 ± 18	66 ± 6
Cys	66.4 ± 1.8	10.9	20.7	29 ± 3	Trace	26 ± 4	19 ± 3
Met	70.4 ± 1.5	6.1	14.2	26 ± 5	4.1 ± 0.6	71 ± 8	16 ± 2
Ile	76.0 ± 1.0	5.9	7.1	63 ± 9	5.8 ± 2.1	154 ± 9	28 ± 3
Leu	78.3 ± 0.9	7.4	6.4	132 ± 14	13.5 ± 4.3	224 ± 18	52 ± 5
Tyr	81.2 ± 0.4	6.8	11.4	59 ± 10	8.8 ± 1.5	114 ± 10	40 ± 6
Phe	83.2 ± 0.7	7.3	9.4	66 ± 10	10.0 ± 2.0	114 ± 6	62 ± 5
Trp	95.7 ± 0.5	6.0	5.3	53 ± 7	2.2 ± 0.5	83 ± 17	8 ± 1
Ea	104.3 ± 0.5	13.4	38.8	–	9.3 ± 1.3	9 ± 1	128 ± 15
Orn	119.6 ± 0.4	8.7	10.3	59 ± 13	5.6 ± 0.9	41 ± 3	5.0 ± 0.1
Lys	122.6 ± 0.4	5.2	7.0	155 ± 41	23.4 ± 4.9	462 ± 67	142 ± 19
His	125.6 ± 0.4	4.4	5.2	83 ± 14	13.3 ± 1.9	77 ± 6	44 ± 6
MeLys	132.1 ± 0.4	11.3	36.7	14 ± 8	1.2 ± 1.1	22 ± 9	–
3-MeHis	134.5 ± 0.3	17.6	15.5	6 ± 2	–	18 ± 8	–
1-MeHis	137.6 ± 0.4	16.1	34.2	7 ± 1	–	14 ± 3	8 ± 7
Arg	175.7 ± 0.3	6.5	7.5	64 ± 12	17.9 ± 5.0	170 ± 13	47 ± 6

[a] The RT (min) for detectable amino compounds not mentioned in the table are: cysteic acid (2.8), glycerophosphoethanolamine (4.3), reduced glutathione (GSH; 22.4), methionine sulphoxide 1, 2 and 3 (32.6, 34.4 and 34.9), α-aminoadipic acid (AAAD; 53.3), glutathione (GSSG; 54.7), dihydroxyphenylalanine (DOPA; 72.2), cystathionine (74.0), norleucine (80.4), β-alanine and β-aminoisobutyric acid (83.2), γ-aminobutyric acid (GABA; 85.0), 5-hydroxytryptophan (5-HTP; 88.4), hydroxylysine 1 and 2 (107.6 and 108.9), ammonia (109.1), homocarnosine and carnosine (133.5), anserine (136.4), α-aminoguanidinopropionic acid (AAGP; 144.4), and putreanine (155.0).

[b] Taurine was co-eluted with glycerophosphoethanolamine, and phenylalanine with β-alanine and β-aminoisobutyric acid. Additional compounds quantitated in rat brain cortex were (mean ± S.D., nmol/g wet weight): reduced glutathione (2014 ± 258), glutathione (199 ± 28), cystathionine (26 ± 4), GABA (2039 ± 145), homocarnosine (53 ± 6), and putreanine (12 ± 3).

To improve the resolution of threonine and serine, glutamate and glutamine, citrulline and α-aminobutyric acid, and cystine and methionine the first 80 min of the chromatogram was run at 0.2 ml/min (*ca.* 70 bar). Thereafter the flow-rate was increased to 0.3 ml/min, giving a working pressure of *ca.* 100 bar at 50°C.

The chromatographic conditions for the separation of the basic amino acids are not optimal. Whereas *ca.* 30 acidic and neutral amino acids are separated within the first half of the run, less than half that number are separated during the second half, and the compounds in the latter part emerge as broader peaks (Fig. 1). A faster increase of the percentage buffer B resulted in a poor separation of ornithine, lysine, histidine and their methylated derivatives.

To evaluate the reliability of the method for making routine measurements of individual amino acids in plasma, the within-assay and between-assay variability were assessed. A pool of human plasma was prepared, divided into aliquots, and stored at −80°C. Eight runs of the plasma pool were made on one day, and another eight runs were made on eight separate days over four weeks. The within-assay C.V. ranged from 3.4 to 17.6%, with a median of 6.2%, and the between-assay C.V. ranged from 2.7 to 39.0%, with a median 10.8% (Table II). A chromatogram of human plasma is shown in Fig. 2.

The linearity was investigated by twice analysing samples containing 50, 100, 500, 1000, 5000 and 10 000 pmol of each amino acid with the fluorimeter at the medium sensitivity setting (the high setting relative to the medium setting increases the sensitivity by approximately a factor of 5). The average peak areas were calculated and plotted against the known concentrations. The response of the system was linear for 32 amino acids from 50 to 5000 pmol or above. Ten amino

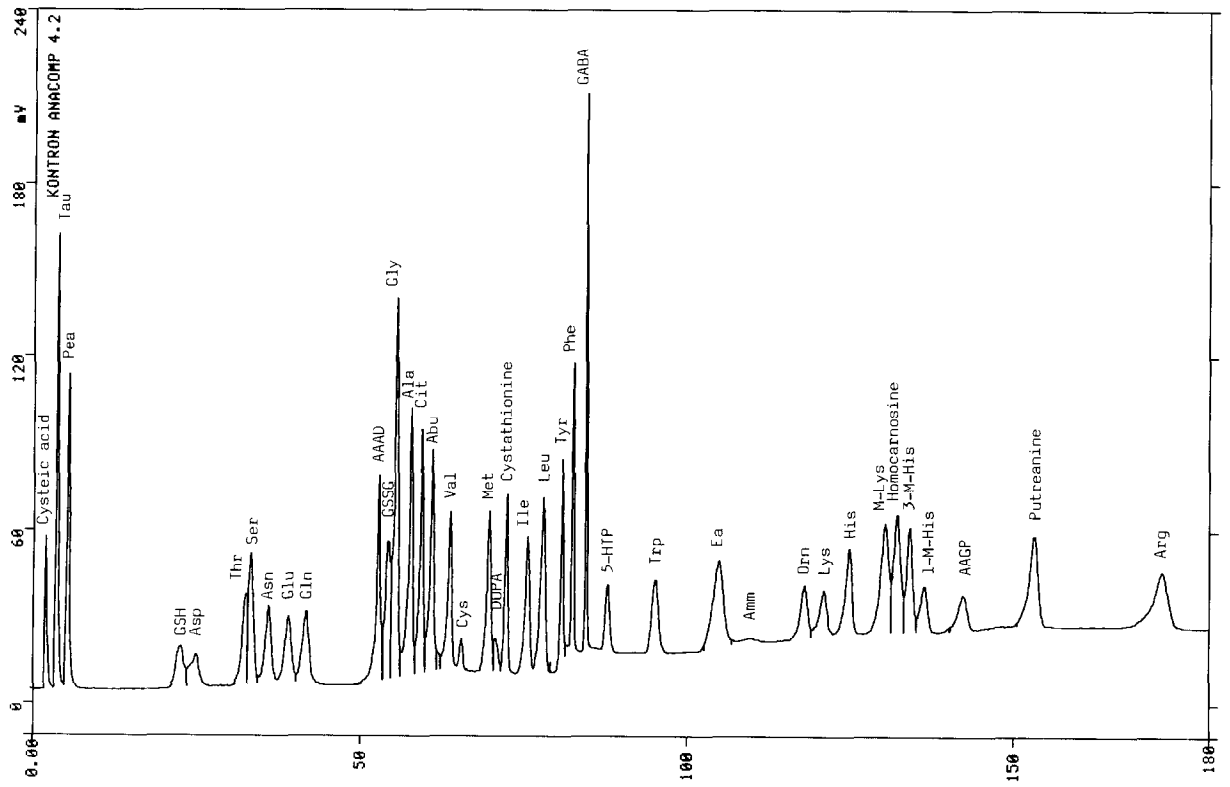

Fig. 1. Elution profile of a synthetic mixture of 40 amino compounds. Abscissa: retention time (min). Ordinate: fluorescence response. Each peak corresponds to 600 pmol.

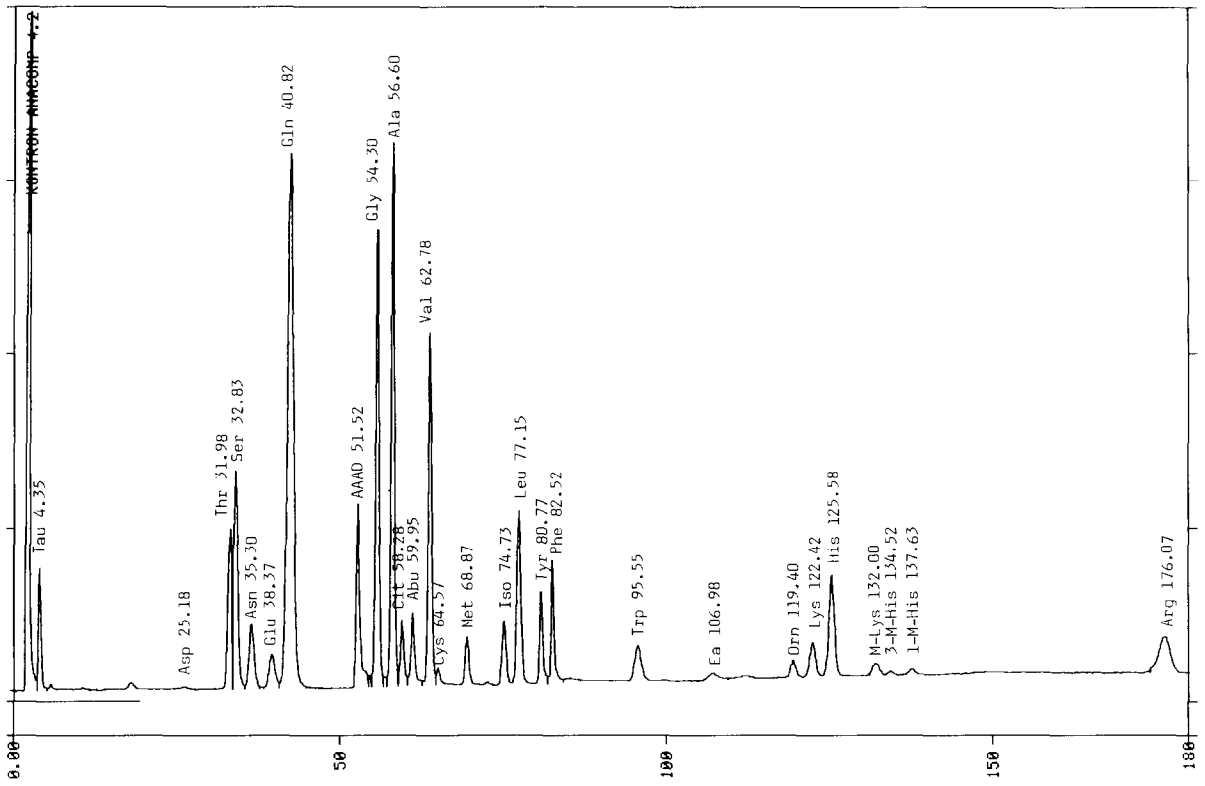

Fig. 2. Typical chromatogram of 7 μl of sulphosalicylic acid-deproteinized human plasma. Abscissa: retention time (min). Ordinate: fluorescence response. The first peak is sulphosalicylic acid.

acids started to deflect at lower levels: taurine, 3 nmol; glycine, 3 nmol; alanine, 4 nmol; citrulline, 2.5 nmol; aminobutyric acid, 4 nmol; phenylalanine, 3 nmol; β-alanine, 2 nmol; aminoisobutyric acid, 2 nmol; GABA, 2.5 nmol; 3-methylhistidine, 4 nmol. The sensitivity of the system in the applied routine setting was estimated to be 3–5 pmol for taurine (C.V. 7.5%, $n = 6$).

The concentrations of individual amino acids in plasma from six fasting, drug-free male subjects, in CSF from six fasting subjects, and in plasma and brain cortex from six fasting, drug-free rats are shown in Table II. Since the response of glutamine, which is present in CSF in higher concentrations than some other amino acids, *e.g.* tryptophan, in some cases went off-scale, separate short runs (65 min) were made with 7 μl of CSF for the quantitation of glutamine relative to α-aminoadipic acid.

There is also a wide range of the amino acid concentrations in brain extract. Most amino acids were quantitated after the injection onto the column of 35 μl of a mixture of 200 μl of brain extract and 50 μl of α-aminoadipic acid solution (200 nmol/ml). For the quantification of the most abundant amino acids, *e.g.* glutamate and GABA, 3 μl of a mixture of 50 μl of brain extract and 50 μl of α-aminoadipic acid solution (1 μmol/ml) were injected onto the column. The buffer gradient profile used for the separation of the amino acids in plasma and CSF (Table I) was not ideal for the separation of the amino acids in rat brain cortex, because glycerophosphoethanolamine co-eluted with taurine, and phenylalanine with β-alanine and β-aminoisobutyric acid.

Generally, the amino acid levels found in this study accord with other findings. However, the present levels for glutamate in plasma and CSF

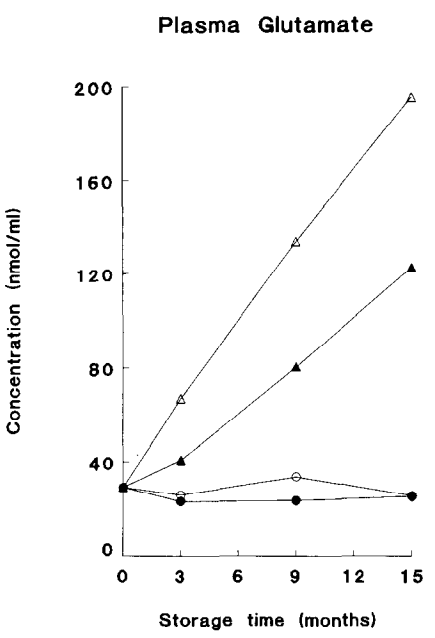

Fig. 3. Influence of storage time and temperature on the concentration of glutamate in human plasma stored on genuine form at −20°C (△) and −80°C (○), and on sulphosalicylic acid protein-precipitated form at −20°C (▲) and −80°C (●)

were lower than the respective mean literature values. Uhe *et al.* [18], who also found lower glutamate levels relative to some other published data, discussed several possible reasons for the discrepancy. One additional explanation could be the instability of glutamine, which decomposes during inappropriate storage and partly is converted into glutamate. The influence of the storage temperature and sample preparation on plasma glutamate levels is shown in Fig. 3. The concentration of glutamate in plasma was stable for more than one year at −80°C, whereas the concentration increased considerably with storage time at −20°C in deproteinized as well as genuine plasma.

No attempt was made to quantitate proline and hydroxyproline, which do not react with the *o*-phthalaldehyde reagent. Addition of hypochlorite as a post-column reagent overcomes the problem of non-reactivity. In a conventional gradient lithium buffer system used to quantitate physiological amino acids, hydroxyproline was eluted between aspartate and threonine, and proline between glutamate and glycine [2].

CONCLUSIONS

A reliable method for quantifying amino acids in plasma, CSF and brain extract by ion-exchange chromatography with post-column derivatization has been reported. The advantages of the method are (1) a robust and stable system that runs automatically and provides data with high accuracy, (2) no preparatory extraction procedures, (3) minimum use of organic solvents, (4) no co-elution of principal physiological amino acids, (5) separation of a larger number of compounds than is usually reported for reversed-phase methods, and (6) cost savings based on component equipment and laboratory-prepared buffers. The drawback of the method relative to reversed-phase chromatography is a considerably longer run time.

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