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DETERMINATION OF AMINO ACIDS BY SEPARATION OF THEIR ION PAIRS WITH DODECYL SULPHATE

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SUMMARY

The ion pairs of amino acids with dodecyl sulphate were separated on a reversed-phase column (Beckman Ultrasphere™ I.P.) using a sequence of eluents that are prepared by mixing 0.2 *M* phosphoric acid (containing 10 m*M* sodium dodecyl sulphate), 0.2 *M* sodium acetate (pH 4.50; containing 10 m*M* sodium dodecyl sulphate) and methanol. Mixtures of the amino acids commonly occurring in tissues, except taurine and related weakly basic amino acids, can be analysed at a rate of 95 min per sample at a sensitivity of less than 50 pmol per amino acid. Elution modes for specific amino acids (α-difluoromethylornithine, γ-vinyl-4-aminobutyric acid, 4-aminobutyric acid, putreanine) and non-essential amino acids that allow higher separation rates are reported.

The method is suitable for fully automated routine amino acid analysis.

INTRODUCTION

Among the various strategies of amino acid determinations by high-performance liquid chromatography (HPLC), separation of ion pairs of the non-derivatized amino acids seems to be not in much use. In a recent review [1] this possibility was barely mentioned, although the high quality of reversed-phase columns and the successful application of this type of method for the separation of biogenic amines [2—4] is in favour of it. In a study of literature we found only four pertinent publications [5—8] of which only one [8] seemed to give satisfactory separations. In our attempt to adopt this method, the low buffering capacity of the eluent proved disadvantageous.

Therefore, an alternative elution system was established. The method described in this paper has been applied to the determination of the amino acids in tissue extracts.

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EXPERIMENTAL

Chemicals

Common laboratory chemicals, including 2-mercaptoethanol and *o*-phthalaldehyde, were from Merck (Darmstadt, F.R.G.). L-Amino acids for the preparation of standard solutions (in 0.1 *M* perchloric acid) were from Sigma (St. Louis, MO, U.S.A.) and sodium dodecyl sulphate (electrophoresis purity reagent) was from Bio-Rad Labs. (Richmond, CA, U.S.A.).

Apparatus

A Varian Vista 5500 liquid chromatograph equipped with a loop injector valve with a 200-μl loop and a Model 8085 autosampler was used. Separations were performed on a thermostated (25°C) column (Beckman Ultrasphere™ I.P.). This column (25 cm × 4.6 mm I.D.) was filled with 5-μm pellicular material with chemically bonded C_{18} groups. The separation column was protected by a guard column (7 cm × 2.1 mm I.D.) filled with Pellicular ODS (C_{18} groups bonded to 37—53 μm particles; Whatman, Clifton, NJ, U.S.A.). The column eluate (flow-rate 1 ml/min) was mixed in a 1:1 ratio with the *o*-phthalaldehyde—2-mercaptoethanol reagent, and after passing through a PTFE coil (1 m × 0.5 mm I.D.) fluorescence was continuously recorded using a Varian Fluorichrome filter fluorimeter (excitation, 345 nm; emission, 455 nm). This fluorescence detector was equipped with a 12.5-μl flow cell. Signals were usually recorded at two sensitivities (Omniscribe, two-channel pen recorder, Houston Instruments, Gistel, Belgium).

Solvents

Aqueous solutions were prepared using tap water distilled over phosphoric acid. Gradients were prepared by mixing the following three solvents. Solvent A: 0.2 *M* phosphoric acid containing 10 m*M* sodium dodecyl sulphate. Solvent B: 0.2 *M* sodium acetate (pH adjusted to 4.50 with acetic acid) containing 10 m*M* sodium dodecyl sulphate. Solvent C: methanol.

o-Phthalaldehyde—2-mercaptoethanol reagent

The *o*-phthalaldehyde—2-mercaptoethanol reagent was prepared by dissolving 50 g boric acid and 31.5 g sodium hydroxide in 1 l water; 3 ml of Brij-35 solution, 3 ml of 2-mercaptoethanol and a solution of 400 mg of *o*-phthalaldehyde dissolved in 5 ml of methanol were added to the borate buffer. Potassium hydroxide could not be used since potassium dodecyl sulphate is precipitated when mixed with the column eluent.

The reagent was stored in dark bottles and used without further precautions, however, not longer than two days.

Tissue preparation and sample application

Tissue extracts were prepared by homogenisation with 10 vols. of 0.2 *M* perchloric acid [10 ml/g of tissue; tissue amounts ranging from a few milligrams to several grams (whole organs)] and centrifugation. The supernatants were first diluted with the same volume of distilled water. Further dilutions were made with 0.1 *M* perchloric acid. Samples were applied on the column in 0.1 *M*

perchloric acid solution in a volume of up to 200 μl which volume did not significantly influence the separations.

RESULTS

The separation of a complex amino acid mixture (Table I) in the form of ion pairs with dodecyl sulphate, using a reversed-phase column, was achieved by a relatively complicated sequence of solvent mixtures which differed both in pH and methanol content (Table II). Owing to the detection reaction with *o*-phthalaldehyde only amino acids with primary amino groups were included in the standard amino acid mixture.

Fig. 1 shows the separation of a mixture of amino acids and related compounds. Taurine, hypotaurine, cysteic acid and related weakly basic amino acids seem not capable of forming ion pairs with sodium dodecyl sulphate and are therefore eluted close to the solvent front. All other common amino acids listed in Table I are sufficiently well separated, as to allow their quantitative determination. D,L-α-Difluoromethylornithine or norvaline can be used as internal standard.

TABLE I

CODE NUMBER AND CAPACITY FACTOR (k') OF THE AMINO ACIDS AND PEPTIDES SEPARATED UNDER STANDARD ELUTION CONDITIONS

Code No.	Amino acid	k'
1	Taurine, hypotaurine, cysteic acid, phosphoethanolamine	0.4
2	Glutamine	3.6
3	Serine	3.8
4	Aspartic acid	4.3
5	Glutamic acid	4.6
6	Glutathion	5.0
7	Threonine	5.4
8	Glycine	5.7
9	Cystine	6.1
10	α-Alanine	6.2
11	β-Alanine	7.1
12	α-Difluoromethylornithine (DFMO)	8.6
13	4-Aminobutyric acid (GABA)	9.4
14	Tyrosine	10.1
15	Valine	10.6
16	Methionine	11.1
17	Norvaline	12.0
18	Ornithine	13.3
19	Lysine	16.6
20	Histidine	17.9
21	Isoleucine	18.4
22	Phenylalanine	21.0
23	Leucine	21.6
24	γ-Vinyl-GABA (4-aminohex-5-enoic acid; GVG)	22.4
25	Homocarnosine	23.8
26	Carnosine	24.3
27	Tryptophan	24.9
28	Arginine	28.0
29	Putreanine [N^1-(2-carboxyethyl)-1,4-butanediamine]	28.8

TABLE II

COMPOSITION OF THE ELUENT FOR THE SEPARATION OF A COMPLEX AMINO ACID MIXTURE

End time: 80 min; equilibration time: 15 min; flow-rate: 1 ml/min; column temperature: 25° C. For solvent composition see Experimental.

Elution time (min)	Percentage solvent		
	A	B	C
0	63	27	10
10	63	27	10
11	40	60	0
20	40	60	0
21	41	56	3
30	41	56	3
31	35	60	5
57	35	60	5
58	0	75	25

Complete separation was achieved within 80 min. Before the next sample can be applied a 15-min equilibration period with the initial solvent mixture is required.

Samples containing amino acids in concentrations ranging between 200 pmol/ml and 10 nmol/ml were separated on three different days and the chromatograms were evaluated by peak height measurements. Even with this simple evaluation procedure, the standard deviation (S.D.) was $\leqslant \pm 5\%$ of the respective mean value for all samples containing 500 pmol or more per ml, although

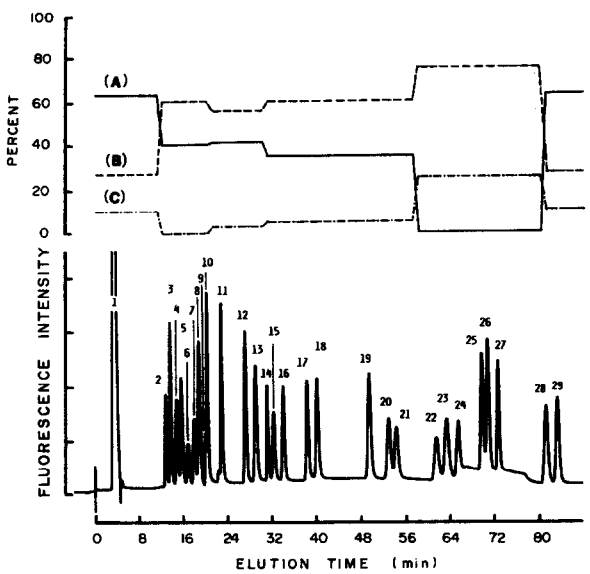

Fig. 1. Gradient composition and separation of the ion pairs with dodecyl sulphate of amino acids (1 nmol) by gradient elution of a reversed-phase column. For the code numbers see Table I; for details of the method see Experimental.

the fluorescence intensity observed after reaction with *o*-phthalaldehyde varies greatly, depending on the structure of the amino acids as can be seen from Fig. 1. Within the mentioned range the relationship between concentration and recorded peak height was linear ($r^2 < 0.99$) for all amino acids.

In Figs. 2 and 3 separations of perchloric acid extracts of mouse brain and liver are shown. They demonstrate the practical applicability of the method. Amino acid concentrations were determined in the brains of CD_1 male albino mice (weighing between 35 and 40 g). The values obtained from these measurements were in good agreement with published data [9, 10], which were obtained by conventional ion-exchange column chromatography. The average standard deviation (for all amino acids) was ± 14.5% of the mean values of a total of nineteen brains. Repeated separations of the same brain extract were performed with a reproducibility indentical to that obtained with standard amino acid mixtures (S.D. $\leqslant$ ± 5% of the mean values; $n = 4$).

If the complete pattern of amino acids is not required, the gradient elution can be interrupted at any point, by changing the solvent composition to 65% B and 35% C so that all following amino acids are rapidly eluted. Shorter runs can

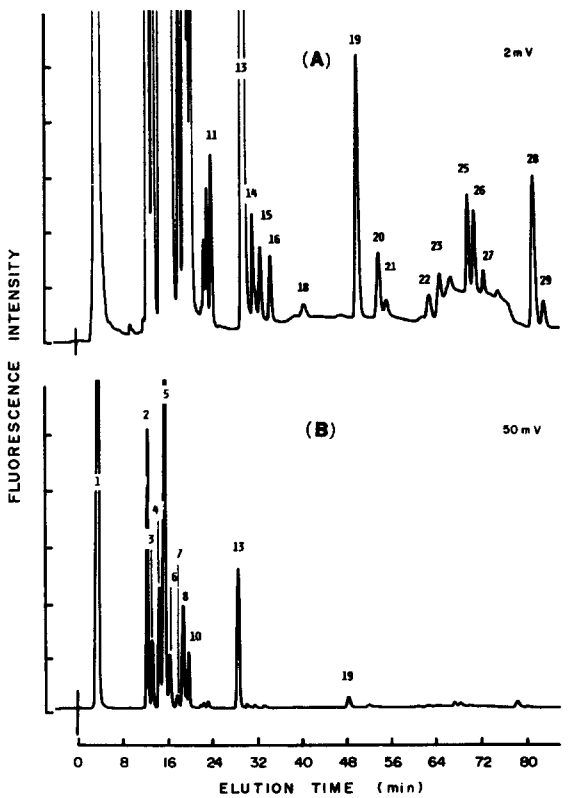

Fig. 2. Separation of the amino acids of a mouse brain extract. A whole brain was homogenised with 10 vols. of 0.2 *M* perchloric acid. The perchloric acid extract was diluted with the same volume of water and a 200-µl aliquot was separated under conditions identical with those shown in Fig. 1. (A) Recording of fluorescence intensity at a recorder sensitivity of 2 mV (full scale); (B) same run as in A, but recorded at 50 mV (full scale). For code numbers see Table I; for details of the method see Experimental.

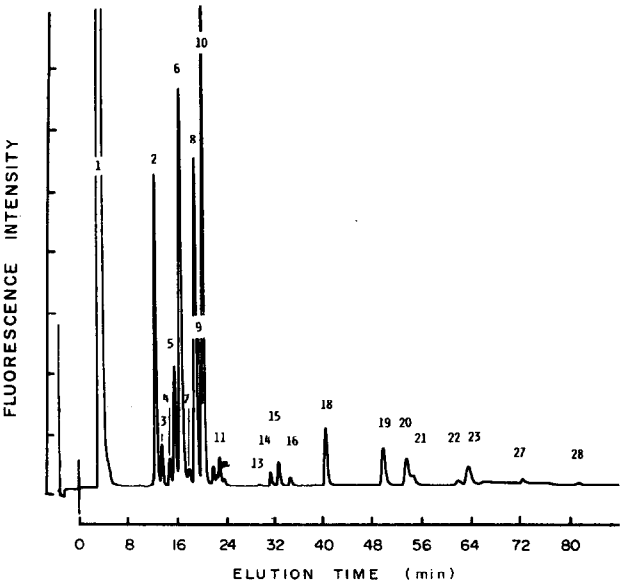

Fig. 3. Separation of the amino acids of a mouse liver extract. The perchloric acid extract was diluted 1:1 with water (see Fig. 2). Separation conditions are the same as those in Fig. 1, recorder sensitivity 50 mV (full scale). For code numbers see Table I.

be achieved by elution programmes which are designed for specific separations.

The determination of the non-essential amino acids is a very frequent requirement. They can be separated by isocratic elution with the following solvent mixture: A = 63%; B = 27%; C = 10%. Glutamine, serine, aspartate glutamate, glutathion, threonine, glycine and with somewhat lower sensitivity β-alanine, GABA and α-alanine can be determined in tissue extracts within 35 min with this elution mode (Fig. 4). Because of their low concentration in tissues amino acids with a capacity factor higher than that of alanine do normally not influence significantly the next separations.

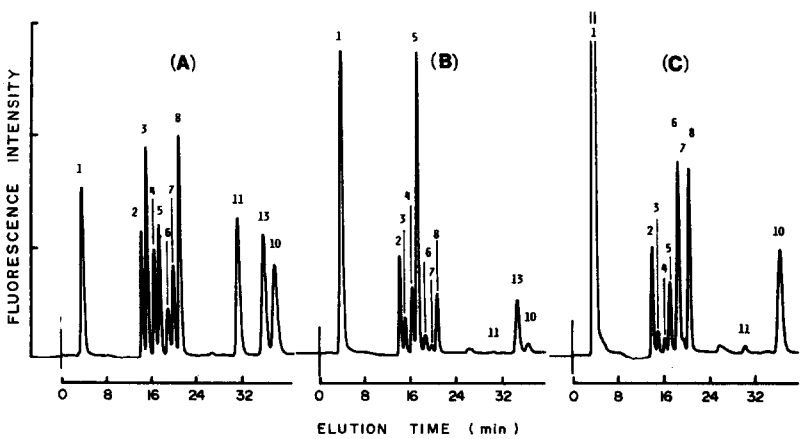

Fig. 4. Isocratic separation of the non-essential amino acids. (A) Standard amino acid mixture; (B) perchloric acid mouse brain extract (1:19 dilution); (C) perchloric acid mouse liver extract (1:19 dilution). Solvent: A = 63%; B = 27%; C = 10%; flow-rate 1 ml/min. For details of the method see Experimental and Results; for code numbers see Table I.

TABLE III

COMPOSITION OF THE ELUENT FOR SENSITIVE GABA DETERMINATION

End time: 25 min; equilibration time: 10 min; flow-rate: 1 ml/min; column temperature: 25°C. For solvent composition see Experimental.

Elution time (min)	Percentage solvent	
	B	C
0	97	3
8	97	3
9	65	35

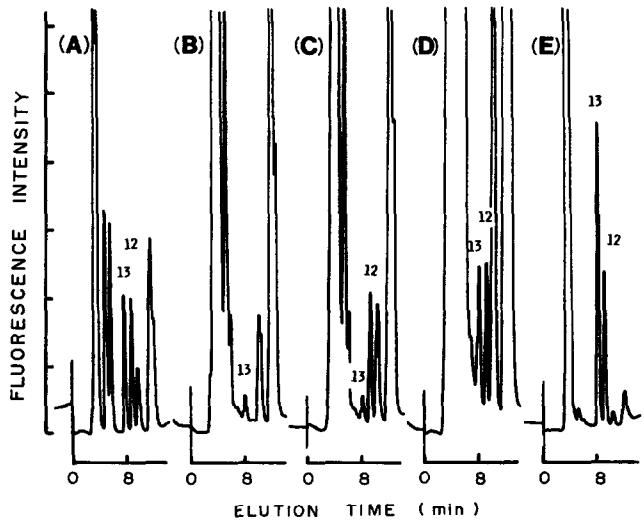

Fig. 5. Elution mode suited for the sensitve determination of GABA and α-difluoromethylornithine. Separation is achieved by elution for 8 min with a mixture of 97% B and 3% C. The more polar amino acids are eluted with 65% B and 35% C. (A) Complete standard amino acid mixture (see Table I); (B) perchloric acid mouse liver extract (1:9 dilution); (C) perchloric acid liver extract (1:9 dilution) with 1 nmol/ml α-difluoromethylornithine; (D) perchloric acid liver extract (1:1 dilution) with 1 nmol/ml α-difluoromethylornithine; (E) mouse brain extract (1:99 dilution) with 1 nmol/ml α-difluoromethylornithine. For details of the separation method see Experimental and Results; for code numbers see Table I.

Owing to peak broadening at isocratic elution mode GABA determinations are of restricted sensitivity with the above-mentioned system. The determination of GABA in amounts smaller than 50 pmol is possible using the binary system described in Table III. In Fig. 5, separations of a brain extract and liver extracts are shown. α-Difluoromethylornithine, the most widely used irreversible inhibitor of ornithine decarboxylase [11], can advantageously be used as internal standard, or, alternatively, the method can be used for the sensitive determination of this drug. The GABA peak in a 1:9 dilution of a mouse liver extract corresponds to about 40 pmol, the α-difluoromethylornithine peak to 200 pmol (Fig. 5). One separation including equilibration with the initial eluent requires 35 min.

TABLE IV

COMPOSITION OF THE ELUENT FOR SENSITIVE DETERMINATION OF PUTREANINE

End time: 40 min; equilibration time: 10 min; flow-rate: 1 ml/min; column temperature: 25°C. For solvent composition see Experimental.

Elution time (min)	Percentage solvent		
	A	B	C
0	33	58	9
18	33	58	9
19	0	65	35

Putreanine is an amino acid uniquely occurring in brain [12]. The gradient described in Table IV is suitable for its complete separation from all other components of extract from brains of mammals and birds. In adult mouse brain 22 ± 3 nmol/g (n = 19), in the brain of a one-day-old chicken 4.3 ± 0.7 nmol/g (n = 4) of putreanine was determined. Using an ion-exchange chromatographic method [13] 17.9 ± 1.9 nmol/g putreanine was found in mouse brain. The same elution mode can also be used for the determination of arginine, and in tissue extracts for ornithine and lysine (Fig. 6). In urine these latter two amino acids were co-migrating with other, not identified components.

γ-Vinyl-GABA is an irreversible inhibitor of 4-aminobutyrate: 2-oxoglutarat aminotransferase [14] with anticonvulsant properties [15]. It is presently in clinical trial as antiepileptic drug and has also great importance as a tool in the study of the GABA neuronal systems. Although it is a close analogue of GABA,

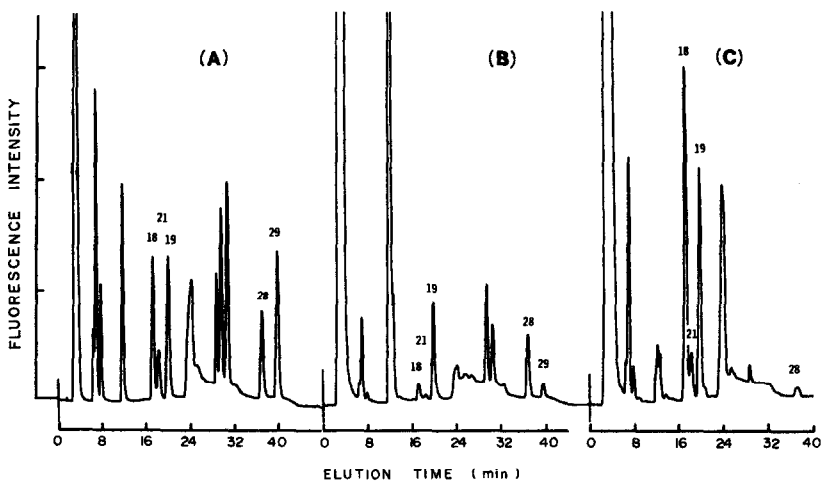

Fig. 6. Elution mode suited for the determination of some basic amino acids. Separation is achieved by a two-step gradient: 0—18 min: A = 33%; B = 56%; C = 11%; 19—35 min: B = 65%; C = 35%. (A) Standard amino acid mixture; (B) perchloric acid mouse brain extract; (C) perchloric acid mouse liver extract. In urine samples ornithine co-migrates with a not yet identified compound. For details of the separation method see Experimental and Results; for code numbers see Table I.

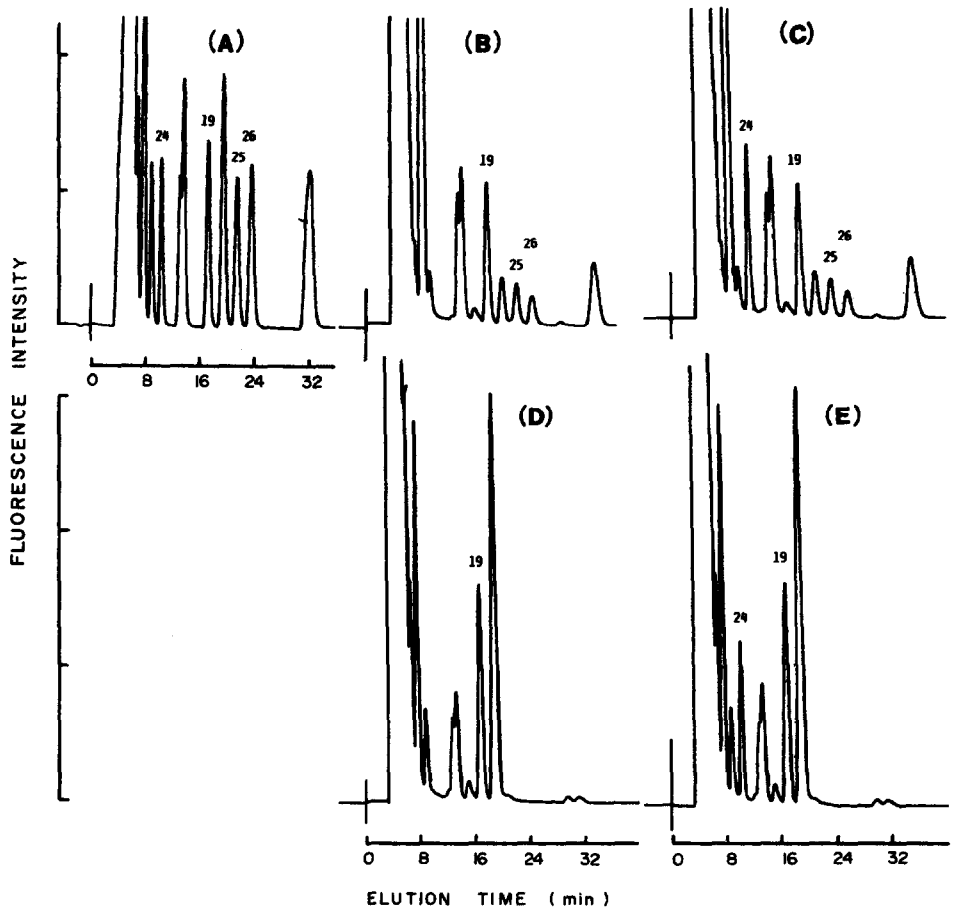

Fig. 7. Elution method suited for the determination of carnosine, homocarnosine, lysine and γ-vinyl GABA. Isocratic elution with a mixture of 70% B and 30% C. (A) Complete standard amino acid mixture (see Table I); (B) perchloric acid mouse brain extract (1:1 dilution with water); (C) same extract as in B with 1 nmol γ-vinyl GABA per 0.1 ml; (D) perchloric acid mouse liver extract (1:1 dilution with water); (E) same extract as in D with 1 nmol γ-vinyl GABA per 0.1 ml. For details of the method see Experimental and Results; for code numbers see Table I.

γ-vinyl-GABA has surprisingly a much higher capacity factor than GABA (Table I). It elutes at a position where, owing to a step in the gradient, the baseline is not flat, so that the standard system is not well suited for its sensitive determination. However, isocratic elution with a mixture of 70% B and 30% C separates γ-vinyl-GABA from all components of brain or liver extracts, as can be seen in Fig. 7. The method allows one to determine about 50 pmol of γ-vinyl-GABA. Lysine, homocarnosine and carnosine are also eluted as uniform peaks and can be determined at the same time, whereas ornithine and histidine and arginine and putreanine, respectively, co-migrate in this system.

DISCUSSION

Methods separating ion pairs of amino acids on reversed-phase columns

resemble in many respects methods employing ion-exchange resin filled columns, with the advantage that the reversed-phase columns do not show volume changes during elution with pH gradients. A practical advantage of both these methods, as compared with methods employing pre-chromatographic derivatization, is the minimal sample preparation and the stability of most amino acids in acidic extracts, which can be directly separated.

The separations described in this paper suggest that our method is a reasonably sensitive and rapid routine method for amino acid determinations in biological materials.

It appears that the retention times of certain amino acids are extremely sensitive to changes of pH and polarity. An example is the difference between the capacity factor of α-alanine in the standard gradient system (Fig. 1) and the isocratic system which is used for the determination of the non-essential amino acids (Fig. 4). While α-alanine elutes in the first system well ahead GABA (k' = 7.1), it appears only after GABA in the second system (k' = 12.1). In contrast, the capacity factor of GABA is similar in the two systems (k' = 9.4 and 11.5, respectively). It is evident from this observation that only devices with very precise pumps, which allow one to form highly reproducible gradients, are suitable for amino acid separations.

In a previous work [8], a simple gradient was pointed out as advantageous. For the separation of complex amino acid mixtures, we needed, however, a complicated sequence of eluents which were prepared by mixing three different components. Admittedly, it is a certain advantage, if a simple device can be used for the solution of a given analytical problem. However, the present state of technical development makes it easy to produce complex gradients in a highly reproducible fashion, so that the usefulness of a separation method is not hampered in principle by a complicated elution pattern. We consider our work as an example of the use of contemporary technical HPLC equipment in an important area. It demonstrates the great practical advantage of gradient formation of three components.

The versatility of the system is demonstrated by determinations of specific amino acids, or groups of amino acids, using simplified gradients and short runs. All examples described in Results have been repeatedly tested or routinely used. Owing to gradual changes of the column characteristics it is necessary to adapt the elution system from time to time by small corrections of the eluent composition.

The retention of the reversed-phase column was sufficiently high for nearly all pertinent amino acid separations. Samples of 200 µl (0.1 *M* perchloric acid) containing acid-soluble material of as much as 9 mg of tissue were routinely applied for the determination of putreanine, without deterioration of the separations.

The sensitivity of the method is limited by the background fluorescence of the *o*-phthalaldehyde—2-mercaptoethanol reagent; the presence of sodium dodecyl sulphate in the eluent prohibits the use of potassium hydroxide for the preparation of the reagent, because of precipitate formation. An improved reagent could increase the sensitivity to the level of methods using precolumn derivatisation with *o*-phthalaldehyde [1].

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