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ION-EXCHANGE CHROMATOGRAPHY OF PHYSIOLOGICAL SULPHUR AMINO ACIDS ON A HIGHLY CROSSLINKED RESIN

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SUMMARY

Improved resolution of sulphur amino acids and related compounds in physiological fluids is obtained on a new Jeol resin, Type RC-2, with 10 % crosslinkage. The relatively high degree of crosslinking with correspondingly long elution times is neutralized by using more periods of high temperature and increasing the number of buffers from four to six for a two-column system. The fully automatic procedure is carried out for 12 h with a mean coefficient of variation of 1 %. A 70 min programme is also developed for the quantitation of cystine and related compounds obtained from treatment of cystinuria with penicillamine.

INTRODUCTION

Since the original method for ion-exchange chromatography of amino acids was published by SPACKMAN *et al.*[1], many factors have been investigated directed to greater resolution and shorter running times. Complete resolution of asparagine and glutamine was achieved by using lithium citrate buffers[2] instead of sodium citrate for acidic and neutral amino acids. This modification also improved the separations of some other amino acids, except for the cystine–leucine region. However, one disadvantage was the high back-pressure (500 p.s.i.) on the top of the column.

The addition of organic solvents to the buffers used for acidic and neutral columns increases the sharpness of some peaks. This has been studied in detail with *n*-propanol for physiological fluids and protein hydrolyzates[3] and for methanol in protein hydrolyzates[4].

The purpose of this work was the further development of an earlier fragmentary programme[5,6] with a new resin, Jeol RC-2, for physiological fluids, with the particular aim of improving separations in the cystine–leucine region. There were two reasons for this. Firstly, to obtain a good standard programme that covers most amino acid metabolites in the increasing numbers of amino acid disorders, with special regard to sulphur amino acids. Moreover, penicillamine treatment of cystinuria[7] contributes to the excretion of three types of disulphides, which are not separated in any standard two-column system. Secondly, treatment of cystinuria must routinely be followed by quantitative determinations of cystine in urine. Therefore, a 70 min programme was developed for the quantitation of cystine and penicillamine-cystine disulphide.

This eliminates the routine use of a standard 6 h programme for the same purpose.

Lithium concentration, pH, temperature and methanol concentration were all studied and the best combination was selected to resolve all amino acids of major interest in physiological fluids. Naturally, a single elution programme cannot be expected to resolve all ninhydrin-positive substances in biological fluids. Even if a good resolution is obtained at first, there will sometimes be a need for minor modifications. Therefore, it is desirable to know some of the parameters that determine the elution positions of the amino acids.

MATERIALS AND METHODS

Reagents

Concentrated hydrochloric acid, *n*-octanoic acid, lithium hydroxide (monohydrate), lithium chloride (monohydrate), trilithium citrate (tetrahydrate) and trisodium citrate (dihydrate) were obtained from B.D.H. Ltd. (Great Britain). Methanol and sodium acetate, both of analytical grade, were obtained from Merck (G.F.R.).

Ninhydrin, thiodiglycol and methyl cellosolve were Pierce (U.S.A.) reagents supplied for amino acid analysis.

Brij 35 (B.D.H. Laboratory Reagent) was prepared as a solution by dissolving 50 g in 100 ml of hot water. Ultrapure water (Millipore Super-Q) was used for all solutions.

Measurement of pH at 25 °

This was carried out with a radiometer pH meter 26 with scale expansion in conjunction with a combined electrode GK 2302C. Standardization was carried out with 0.05 *M* potassium hydrogen phthalate, which has a pH of 4.01 at 25 °.

Buffer compositions

Buffer compositions are given in Tables I and II. Sodium citrate buffers of pH 4.30 and 5.45 were used for the basic column according to BENSON AND PATTER-

TABLE I

LITHIUM CITRATE BUFFERS

Property	*Values for different buffers*				
pH at 25 °	2.20	2.78	3.05	3.80	4.40
Lithium concentration (*M*)	0.3	0.24	0.26	0.30	0.30
Citrate concentration (*M*)	0.1	0.050	0.065	0.100	0.100
Methanol concentration (%)	—	8	6	—	—
Trilithium citrate tetrahydrate (g)	28.2	70.0	92.0	141.0	141.0
Lithium chloride monohydrate (g)	—	27.5	20.0	—	—
Thiodiglycol (ml)	5	5	5	5	5
Brij-35 solution (ml)	—	10	10	10	10
Concentrated HCl (ml)	26	53	38	74	55
Octanoic acid (ml)	0.1	0.5	0.5	0.5	0.5
Methanol (ml)	—	400	300	—	—
Final volume (l)	1	5	5	5	5

TABLE II

SODIUM CITRATE BUFFERS

Property	*Values for different buffers*		
pH at 25 °	2.97	4.30	5.45
Sodium concentration (*M*)	0.35	0.38	0.35
Trisodium citrate dihydrate (g)	171.5	186.3	171.5
Brij-35 solution (ml)	10	10	10
Concentrated HCl (ml)	112	73	27
Octanoic acid (ml)	0.5	0.5	0.5
Final volume (l)	5	5	5

SON[8], and pH 2.97 buffer for the short cystine–methionine programme. The lithium citrate buffers were used for acidic and neutral amino acids. The pH 2.2 buffer was used as the diluting and application buffer for both columns. For practical purposes, the buffers were made five times concentrated. Only 70% of the hydrochloric acid was added to the concentrate. It is necessary to filter all buffers through a Millipore 0.45 μm filter, especially the lithium buffers and sodium acetate buffers for ninhydrin preparation.

Very thorough mixing was necessary in order to obtain methanol and octanoic acid in solution. It should be stressed that the final pH adjustment must be carried out very carefully to within 0.005 pH units from the required value. The buffer bottles on the analyzer were protected against atmospheric ammonia with citric acid tubes.

Ninhydrin reagent

The reagent according to SPACKMAN *et al.*[1] was used. The reagent was stored in pure nitrogen at 0.25 kp/cm^2 and kept in a refrigerator at 6–8 °.

Resin

The resin for both the acidic–neutral and basic columns was Jeol Type RC-2 with a bead diameter of 10–12 μm and 10% crosslinkage. They were pre-treated with 2 *M* hydrochloric acid, water, 2 *M* lithium hydroxide or 2 *M* sodium hydroxide until the filtrate was colourless. All solutions contained Brij 35. The column was packed in starting buffer by free sedimentation for 4–10 h. The columns were therefore elongated with a plastic tubing of I.D. 13 mm. The dimensions of the acidic–neutral and basic columns were 460 mm and 260 mm, respectively, and I.D. 8 mm.

Analyzer

A Jeol 5-AH analyzer was used in these studies. A 2 mm light path and three-times scale expansion were used on the recorder, but this sensitivity can be increased fifteen times with perfect results. Of the eight buffer connections on a rotary valve for the acidic–neutral column four were used for lithium buffer solutions and one for lithium hydroxide (Fig. 1). The remaining three positions were used for two hydrolysate buffer solutions and one for sodium hydroxide. Corresponding connections were used with the basic column, but one extra connection was also available for the pH 2.97 buffer of the short cystine–methionine programme. The outlets of one

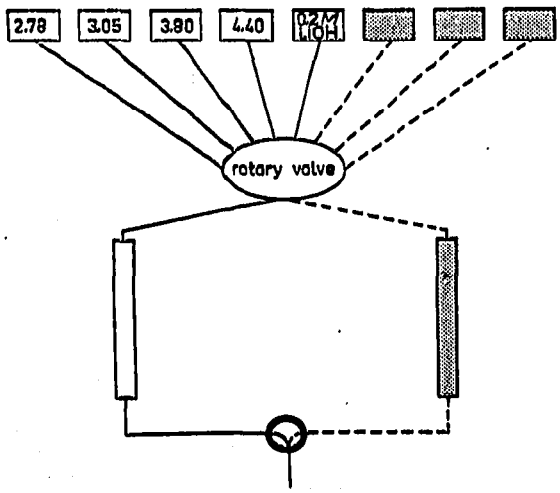

Fig. 1. Illustration of the buffer selection and column outlet for combined physiological and hydrolysate system. The dotted areas represent buffer positions and the column for hydrolysates.

physiological and of one hydrolysate column were connected to a three-way valve, the outlet of which led to the detection pump and mixing manifold. Two different two-column systems for physiological and hydrolysate analyses, respectively, could readily be used on the same instrument with convenient day-to-day flexibility. The risk of air bubbles entering the tubing system was also eliminated in the same way. Calibration of the sample coils at 4° was carried out by slowly injecting 1.2 ml of tritiated water, of which approximately 0.8 ml was left in the coil. The rest of the system was rinsed with 10 ml of distilled water after changing to a new coil position. The tritiated water was eluted with 10 ml of an ordinary buffer for 10 min. The activity was determined in a Packard liquid scintillation counter with an internal standard using BRAY's solution[9].

Sample preparation

Pierce amino acid calibration mixture, which contains 2.5 μmoles/ml of seventeen amino acids, was diluted with pH 2.2 buffer to a final concentration of 0.125 μmole/ml. Most of the other non-protein amino acids were purchased from Calbiochem, Los Angeles, Calif. The final dilution was approximately 0.125 μmole/ml with the following exceptions: urea 0.400, hydroxyproline 0.250, citrulline 0.060, cystathionine 0.050, β-alanine 0.050, β-aminoisobutyric acid 0.600, homocystine 0.060, tryptophan 0.100, carnosine 0.200 and ε-aminocaproic acid 0.300 μmole/ml. The stock solutions were kept at −70°.

Urine

Urine was de-ammoniated by evaporation at alkaline pH (ref. 8).

Plasma

Plasma was de-proteinized with sulphosalicylic acid (50 mg/ml).

Evaluation

The chromatograms were evaluated with a two channel JLC DK integrator.

Procedure

The instrument was furnished with an automatic sample selector, six samples of 0.8 ml for each column. The sample load, which was usually 1.2 ml in pH 2.2 citrate buffer, was slowly injected with a 2 ml syringe. A small air bubble was first injected from the top of the syringe and the appearance of this bubble at the outflow was taken as ensuring adequate sample loading. The sample selector was changed to the next position before the syringe was removed. The inlet and outlet of the system were then rinsed with 10 ml of water before the next sample loading.

Two buffer pumps were used and each pump system could be programmed for an automatic buffer change and regeneration of the column after each cycle. The regeneration process includes 20 min with 0.2 *M* lithium hydroxide followed by 40 min with pH 2.78 buffer for the acidic–neutral column and 20 min with 0.2 *M* sodium hydroxide followed by 40 min with pH 4.30 buffer for the basic column. The flow-rates of the two columns were 0.83 and 1.22 ml/min, respectively.

The cystine–methionine program was performed on the basic column at pH 2.97, 55 ° and 1.22 ml/min.

The ninhydrin reaction was performed at 95 °. A constant volume of 0.42 ml/min was withdrawn from the eluate with a sample pump and mixed with 0.21 ml/min of ninhydrin. The complete elution programme, including the temperature change, is given in Table III.

TABLE III

ELUTION PROGRAMMES

Analysis	*Time (min)*	*Temperature (°C)*	*Buffer (pH)*	*Buffer change, appearance*
Acidic–neutral	0	36	2.78	—
	95	—	3.05	After glutamine
	100	55	—	—
	165	36	—	—
	290	—	3.80	In front of cystathionine
	380	55	—	—
	398	—	4.40	After phenylalanine
	460	36	0.2 *M* LiOH	—
Basic	0	36	4.30	—
	100	55	—	—
	165	—	5.45	After tryptophan
	250	—	0.2 *M* NaOH	—

RESULTS

General

The analysis of a synthetic mixture composed of fourty-four amino acids and related compounds is shown in Figs. 2 and 3. The most apparent difference in comparison with other systems is the improved separation in the cystine–methionine–cystathione–leucinine region. The noticeable separation of threonine and serine is caused by the methanol in the first buffer. Another advantage is the inclusion of homocystine in the standard acidic–neutral programme and the complete separation of tryptophan. This amino acid is often omitted in many programmes or is exposed

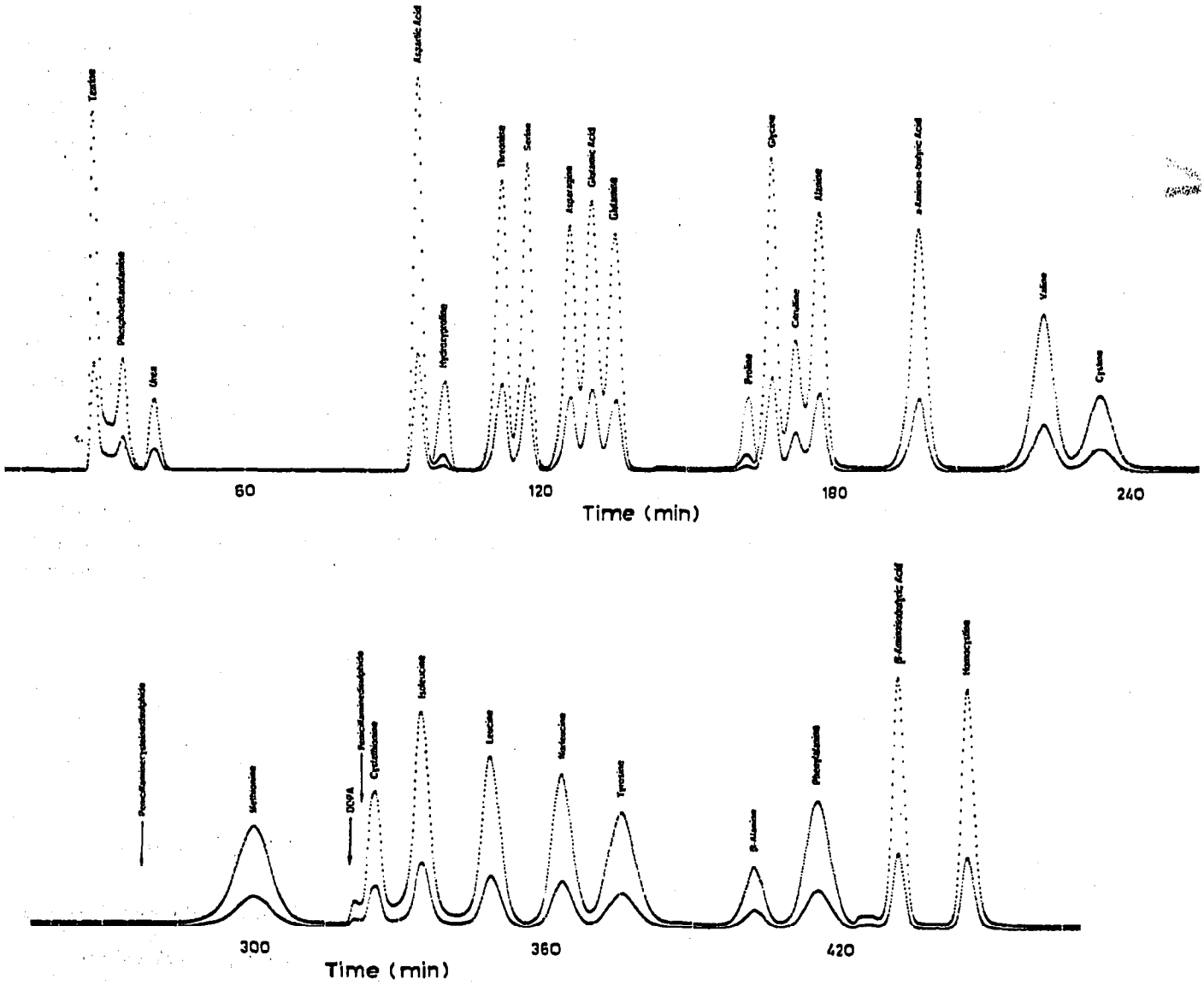

Fig. 2. Analysis of a synthetic mixture of amino acids and related compounds on a 46 cm resin column for acidic and neutral components.

to interference by creatinine, carnosine or the internal standard α-amino-β-guanidopropionic acid. As we also had problems with the separation of this standard and tryptophan, ε-aminocaproic acid was used as the internal standard. This related compound has an elution time rather close to that of arginine and gives a sharp well defined peak, which is necessary for an internal standard. Norleucine is included in the chromatogram more as an internal standard for hydrolysates and not for physiological fluids, as naturally occurring mixed cystine–homocystine disulphide appears at the same elution volume.

The whole programme is completed within 12 h. The pressure on the top of the column is 20 kg/cm² (300 p.s.i.) for both the acidic–neutral and the basic columns. As the resin has a relatively high degree of crosslinking, the elution times are prolonged, although the resolution is remarkably good. To neutralize this effect

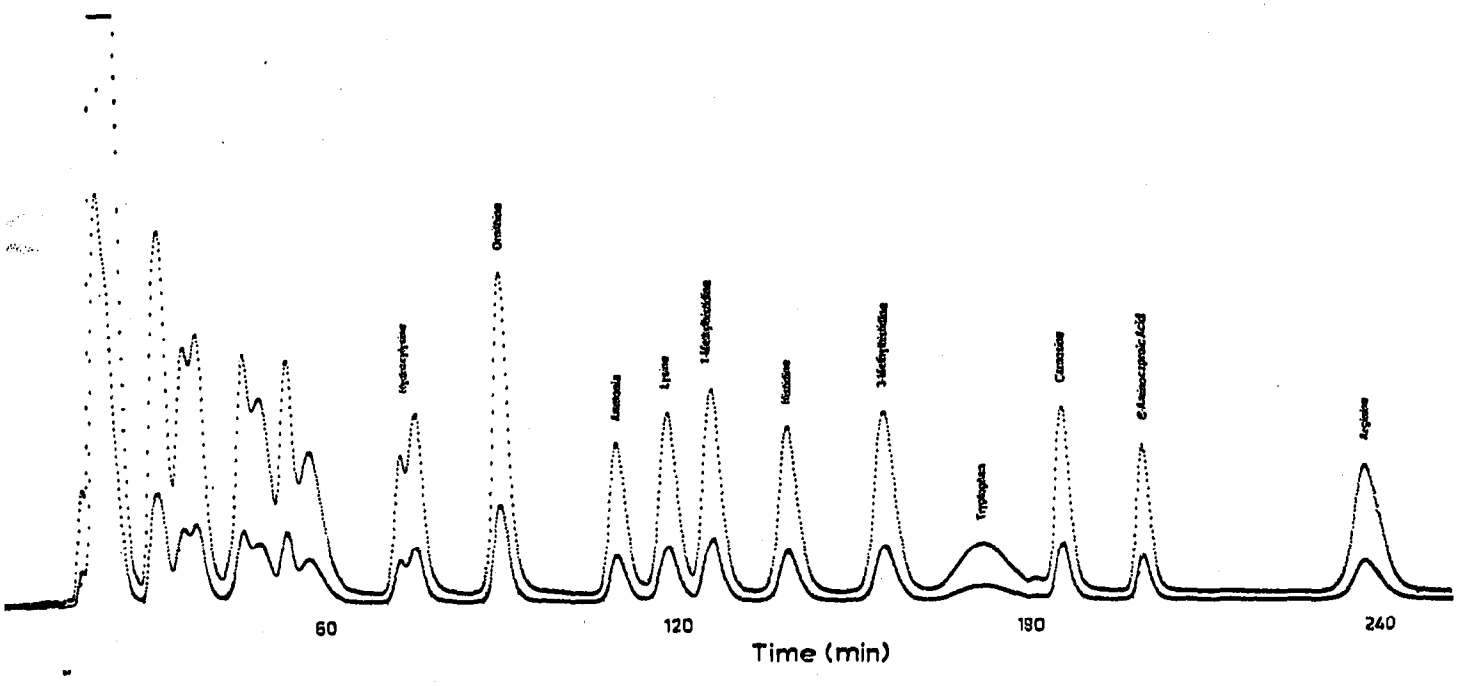

Fig. 3. Analysis of a synthetic mixture of amino acids on a 26 cm resin column for basic components.

on the acidic–neutral column, four buffers instead of two are introduced. The last buffer, of pH 4.40, is necessary only to obtain adequate elution times for β-amino-isobutyric acid and homocystine. The advantages of this procedure are a gain of 80 min and sharper peaks.

Effect of pH, temperature and methanol concentration on the 12 h programme

In general, an increase of ionic strength, pH, temperature and methanol concentration decreases, within limits, the elution times of all amino acids. Table I gives the composition of the lithium buffers. All the buffer changes are located so that they are visible and do not interfere with any peaks. It is also important to allow time for the development of a new baseline level, especially if an integrator is used for the evaluation.

The first compounds, taurine, phosphoethanolamine, urea, aspartic acid and hydroxyproline, are almost independent of pH and temperature. Threonine and serine are dependent on methanol concentration and temperature; 8% and 36°, respectively, give the best results. Methionine sulphone, which occurs in stale urine, comes just before the threonine peak. The separation of asparagine, glutamic acid and glutamine is achieved most successfully with the lithium buffer systems. The position of glutamic acid is very dependent on the pH of the first buffer. We obtained the best results at pH 2.78; with a more acidic pH glutamic acid elutes together with glutamine, and with a more basic system it elutes together with asparagine. The buffer shift at 95 min gave a disturbance in the baseline just after glutamine. Too early a change of buffer results in poor separation or none. Proline is methanol-sensitive; the higher the concentration, the shorter is the elution time. A 6% concentration is satisfactory. In the glycine, citrulline and alanine region, citrulline is remarkably

sensitive to pH and temperature. With a lower temperature and acidic pH it can be eluted after alanine, but in that event the whole group will be retarded. This problem can be overcome by a suitable period at 55 °. Alkaline pH or a temperature of 55 ° for a longer time moves citrulline towards glycine, and a more acidic pH and too short a period at 55 ° moves it towards alanine. No deamination of glutamine or conversion to pyrrolidonecarboxylic acid can be demonstrated because of the period at high temperature. The relative elution time of valine is independent of pH and temperature. Cystine is one of the most sensitive indicators for correct pH adjustment. Acidic pH increases the elution time, and alkaline pH shortens it relatively to valine. At pH 3.10 cystine and valine move together.

Methionine is eluted 60 min after cystine. This period can be shortened by earlier changes of buffer and temperature, but will be accompanied by incomplete separation of methionine and cystathionine and of β-alanine and phenylalanine. The wide area around methionine permits the complete resolution of compounds excreted during the treatment of cystinuria patients with penicillamine. A minor part of the penicillamine is excreted as penicillamine disulphide, which has the same elution volume as dihydroxyphenylalanine (DOPA).

Isoleucine, leucine, norleucine and tyrosine are always separated completely without any problems. β-Alanine and phenylalanine are mainly pH- and temperature-dependent. A more alkaline pH is desirable to shorten the elution time of the last amino acids, but at pH 3.95 β-alanine and phenylalanine already have a tendency to elute together; pH 3.80 is satisfactory. Too early a shift to 55 ° will shorten the elution time of phenylalanine so that it elutes together with β-alanine. β-Aminoisobutyric acid and homocystine separate without any problems. A fourth buffer can be introduced in order to decrease the elution time on the chromatogram.

In the basic column, hydroxylysine, ornithine, ammonia, 1-methylhistidine, histidine and 3-methylhistidine are relatively independent of pH. As hydroxylysine always occurs in small concentrations in plasma and urine, γ-aminobutyric acid is partially separated. At pH 4.45, these two amino acids are separated completely. Lysine is the most pH-sensitive amino acid of the basic system. At pH 4.25, lysine is eluted together with 1-methylhistidine. A more alkaline pH will move it towards ammonia, and at pH 4.45 lysine is completely separated in front of ammonia. Tryptophan is mainly temperature-dependent. The earlier the changes of buffer and temperature, the shorter the elution time will be. The appearance of the buffer change must be located just after tryptophan. The change to 55 ° at 100 min is critical for the shape of the tryptophan peak and its separation from carnosine.

Creatinine is eluted together with carnosine after tryptophan. Creatinine has an extremely low colour index, so that carnosine can be quantitated with sufficient accuracy. The more alkaline the pH, the shorter are the elution times for the internal standard ε-aminocaproic acid and arginine.

Cystine–methionine programme

Fig. 4 shows the separation of cystine, valine, methionine and DOPA on the basic column at pH 2.97 at 55 °. Penicillamine-cystine disulphide elutes partially separated from valine in this system. The urine is often diluted ten times and as valine excretion is normally significantly low, the mixed disulphide can also be estimated at the same time.

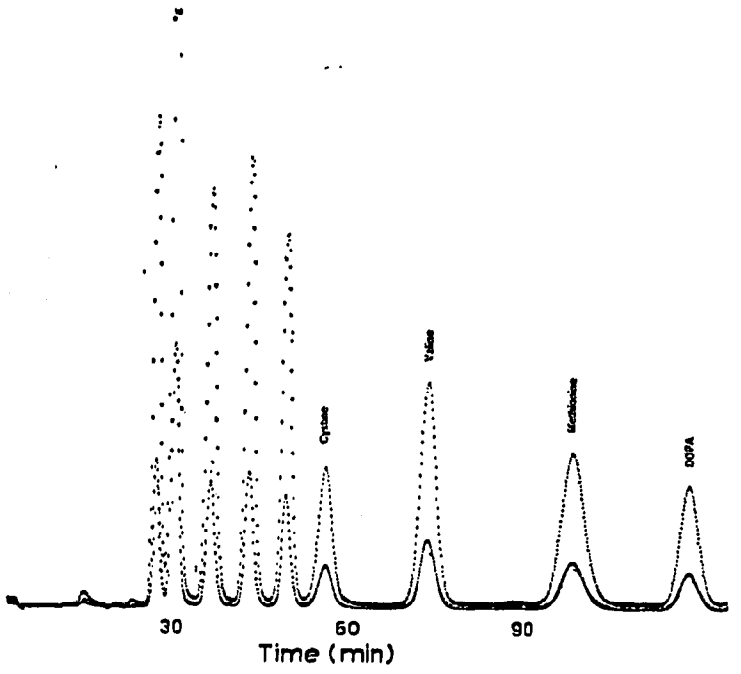

Fig. 4. Short programme for cystine and related compounds on a 26 cm column at pH 2.97 and 55°

Precision of the system

To obtain an estimate of the precision of the system, including the integrator, the coefficient of variation of each peak was calculated from the mean value and standard deviation for four consecutive runs with a synthetic mixture. The coefficients of variation varied from 0.2 to 2.3%, with a mean value of 1.0% if the glutamine value is excluded. The glutamine yield decreased by 10% at 40 h when stored at 4° in the sample collector. As no increase in glutamic acid was obtained,

TABLE IV

COEFFICIENT OF VARIATION DETERMINED AFTER FOUR CONSECUTIVE RUNS FOR 48 h

Substance	*Coefficient of variation* (%)	*Substance*	*Coefficient of variation* (%)
Aspartic acid	1.1	Isoleucine	0.8
Hydroxyproline	0.4	Leucine	0.5
Threonine	0.6	Tyrosine	1.1
Serine	1.0	β-Alanine	2.3
Asparagine	1.6	Phenylalanine	0.7
Glutamic acid	1.0	β-Aminoisobutyric acid	0.9
Glutamine	5.2	Homocystine	1.2
Proline	0.7	Hydroxylysine	1.7
Glycine	1.2	Ornithine	1.0
Citrulline	1.8	Lysine	0.6
Alanine	1.2	1-Methylhistidine	0.2
α-Amino-*n*-butyric acid	0.5	Histidine	1.5
Valine	0.5	3-Methylhistidine	1.0
Cystine	0.6	Tryptophan	1.2
Methionine	0.3	Carnosine	0.9
Cystathionine	1.2	ε-Aminocaproic acid	1.2
		Arginine	1.2

the explanation must be the conversion of glutamic acid into pyrrolidonecarboxylic acid. The elution times are always constant to within ± 1 min.

DISCUSSION

The chromatographic behaviour of amino acid compounds in physiological fluids has been studied on a sulphonated polystyrene resin with 10 % crosslinkage, which is one of the densest resins available. The peak width increases or the peak height decreases as the extent of crosslinking increases. Peak separation is improved as crosslinking is increased, but the elution time increases proportionally because of the longer time needed for equilibrium between the stationary and mobile phases[10]. The exchange capacity on a volume basis is increased and makes a more efficient column. With a highly crosslinked resin, there is no significant change of volume when the ionic strength or methanol concentration is increased.

The disadvantage of the long elution time can be neutralized by a more uniform and smaller particle size, which occurs in RC-2 resin. More periods at higher temperature and an increase in the number of buffers from four to six also counteract the slow reaction of the RC-2 resin. The final gain is an excellent resolution, especially of sulphur amino acids.

The Jeol 5-AH instrument is furnished with eight buffer connections for each column, which facilitates the use of four instead of two lithium buffers for the acidic–neutral column system. It also permits a reasonable day-to-day flexibility if protein hydrolyzates have to be analysed on the same instrument.

Small differences in crosslinkage give different chromatographic patterns with the same combination of pH, ionic strength and temperature. LONG AND GEIGER[11] found a different effect with 7 % and 8 % crosslinking. They obtained good resolution by a final resin mixture with 30 % of the 7 % crosslinked resin and 70 % of 8 % crosslinked resin. The difference in the elution times for arginine between the two types of resin was as much as 1 h. HAMILTON[10] also found that of six different resins, all nominally 8 % crosslinked, none gave exactly the same separations with the same buffer and temperature conditions. From the literature, it also seems that manufacturing conditions can hardly ever be controlled so effectively that different batches of a given resin will be identical. A knowledge of the fundamental parameters of good resolution is therefore valuable in studying complex fluids. There is no single elution programme that resolves all the possible ninhydrin-positive components that can be expected in the increasing numbers of amino acid disorders.

Tryptophan is an essential amino acid that is important in nutrition studies. In most published systems, it is often not separated from creatinine or carnosine, with a few exceptions[12,13]. Although sulphosalicylic acid is superior to picric acid as protein precipitant, 20 % lower values for endogenous levels of tryptophan are obtained compared with fluorimetric estimations. Complete release from the albumin–tryptophan complex can be obtained with trichloroacetic acid as precipitant[12]. However, to avoid the formation of bubbles in cuvettes, the ninhydrin and the column eluant must be pumped to the drain during the first 15 min of the run.

Ethanolamine cannot be separated from ammonia in this system. ATKIN AND FERDINAND[3] suggested that propanol should be added to the buffer to overcome this problem.

Glutamine is often very unstable, leading to errors in quantitation. Glutamine is rapidly converted into pyrrolidonecarboxylic acid, depending on time and temperature. This conversion proceeds even at −20°.

In contrast to PERRY AND HANSEN[14], we have not found any loss depending on a temperature increase from 36° to 55° for 20–25 min before elution. On the contrary, the storage of sample in the automatic sample selector at 4° causes a gradual conversion of glutamine into pyrrolidonecarboxylic acid. This explains the relatively high coefficient of variation. The increased separation of sulphur amino acids is not only applicable to the control of the penicillamine treatment of cystinurias, but the problems of cystathioninuria and homocystinuria will also be easier to solve.

The procedure described is not the most rapid one. Therefore, we prefer to carry out the first urinary screening of amino acid disorders by high-voltage electrophoresis[15] at two different pHs and sometimes simultaneously with thin-layer chromatography of plasma amino acids[16]. Complete ion-exchange chromatography is performed when unclear patterns are obtained. The final analysis is therefore performed with high resolution and precision. This sequence is cheaper and less time-consuming than running a 5.5 h programme[17] with inadequate resolution as a screening programme. Good resolution in a short programme of most of the physiological standard amino acids of major interest is often hard to attain. The new automatic instruments that can be run for 48 h or longer allow a 12 h programme to be used for physiological runs.

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