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CONSTRUCTION OF AN ION-EXCHANGE AMINO ACID ANALYSER KIT FOR USE WITH HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY APPARATUS

M. GRIFFIN*, J. LEAH, N. MOULD and G. COMPTON

Department of Life Sciences, Trent Polytechnic, Clifton Lane, Nottingham NG11 8NS (U.K.)

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SUMMARY

A conventional ion-exchange amino acid analyser kit has been simply and economically constructed for use with existing high-performance liquid chromatography (HPLC) apparatus. Sequential elution conditions are fully programmable by virtue of a 32K BBC microcomputer interfaced with an elution buffer selection valve and a thermostatically controlled column. Post-column derivatization with *o*-phthalaldehyde-2-mercaptoethanol reagent enables fluorimetric detection at the picomole level. The system enables sensitive amino acid analysis of complex mixtures to be carried out by clinical and research laboratories who already possess HPLC apparatus but whose sample turnover does not merit purchase of a dedicated fully automated analyser.

INTRODUCTION

A question which confronts many laboratories when in the process of either updating or purchasing for the first time an instrument for the quantitative analysis of amino acids, is whether to purchase a fully dedicated instrument or one which has the versatility to be used for other purposes. The relative merits of these have been reviewed previously [1,2].

Such a choice is now available because of the recent developments in amino acid analysis using reversed-phase high-performance liquid chromatography (HPLC) as an alternative to the more conventional cation-exchange systems [3].

Since amino acids are polar compounds of diverse charge their interaction with the hydrophobic stationary phase has to be promoted in some way to allow effective resolution of complex mixtures. One technique of increasing amino acid retention is to use ion-pair systems [4] and methods for the separation of amino

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acids using this technique have been reported [5]. However, by far the most commonly employed technique for reversed-phase HPLC analysis of amino acids involves the use of precolumn derivatisation. In this method amino acids are reacted to yield a hydrophobic derivative which not only provides adequate interaction with the column stationary phase but also provides a means of detection.

A number of methods combining precolumn derivatisation and reversed-phase HPLC for the separation of amino acids have now been reported. Reagents used include dansyl chloride [6], phenylthioisocyanate [7], 9-fluorenylmethyl chloroformate [8] and *o*-phthalaldehyde–2-mercaptoethanol (OPA–2-ME) [9–11]. Each system has its advantages and disadvantages but in general derivatisation of amino acids with OPA–2-ME is probably the most versatile when considering ease of derivatisation, method of detection and complexity of columns and gradients required for efficient separation of amino acids.

However, although reversed-phase systems have the ability to provide rapidity of separation and sensitivities in the femtomole range they are limited in the number of components which can be separated when dealing with complex mixtures.

For the analysis of complex mixtures such as physiological fluids, a dedicated ion-exchange system is generally required. This system is very costly, has little versatility and in most laboratories can only be justified if a large number of samples on a continuous basis are to be analysed.

The need is therefore for a versatile system which can be used for both reversed-phase and ion-exchange separation of amino acids and peptides. We report here a system which for a small capital outlay to existing HPLC users, provides the means of using both reversed-phase and ion-exchange chromatography for the analysis of amino acids.

EXPERIMENTAL

Reagents

Amino acid standards, OPA, 2-ME and Brij 35 were purchased from Sigma (London, U.K.). Physiological standards, DL-norleucine, lithium citrate diluent buffer (pH 2.2) and lithium citrate Picobuffer system IV, were purchased from Pierce (Chester, U.K.). Ion-exchange resin DC-4A (sulphonated polystyrene 8% cross-linked with divinylbenzene) supplied in the sodium form from Dionex (Hampshire, U.K.) was converted to the lithium form as instructed by Benson [12]. Analar grade methanol, boric acid and potassium hydroxide were purchased from BDH (Poole, U.K.).

OPA-2-ME reagent used for derivatisation of amino acids was made up as follows: 50 g of boric acid, 44 g of potassium hydroxide and 3.5 ml of Brij 35 were dissolved in double-distilled water, adjusted to pH 10.4 and made up to 1000 ml. A 600-mg amount of OPA was dissolved in 7.5 ml of methanol and 5.0 ml of 2-ME and the solution was added to the boric acid buffer. The reagent was vacuum-filtered through 0.22 μm Durapore filters (Waters Assoc.), degassed and stored

When the system was converted to amino acid detection by reaction with ninhydrin, the reagent was made up by the method of Moore and Stein [13] and contained in a total volume of 1000 ml: 750 ml methyl cellosolve, 250 ml of 4.0 *M* sodium acetate buffer (pH 5.5), 20 g of ninhydrin and 0.4 g of stannous chloride.

Chromatography

An M-45 pump (Waters Assoc.) was employed to pump OPA–2-ME reagent to a post-column low-volume mixing manifold (Waters Assoc.) at a flow-rate of 0.2 ml/min from which a 2-min reaction time was permitted prior to fluorescence detection. A Perkin-Elmer Model 1000 m fluorimeter fitted with a 25-μl flow cell, 339-nm interference filter and an emission wavelength of 455 nm was used for detection. Peak areas were determined using a Spectra-Physics SP 4270 integrator. The buffer pump used at a flow-rate of 0.2 ml/min was a Waters Type 501, fitted with a Rheodyne 7125 injector and 100-μl sample loop. A stainless-steel column with an I.D. of 0.3 cm was used to support the DC 4A ion-exchange resin (final packed dimensions 35 cm$\times$0.3 cm I.D.). A purpose built glass jacket surrounded the column to which water was supplied by a Churchill circulator (Chemlab Instruments, Essex, U.K.).

Elution buffers were stored in stoppered air-tight glass containers kept under a nitrogen pressure of 0.35 bar. Buffer reservoirs were connected to a Rheodyne pressure-regulated selection valve using PTFE tubing (Anachem) connected by flanged connectors (Pierce).

A BBC Model B microcomputer was used fitted with disk drive (Acorn) and interface as described in the text.

Preparation of physiological samples for amino acid analysis

Preparation methods were those described by Benson [12]. Deproteinisation was performed by vortex-mixing 0.8 ml of urine with 0.2 ml of sulphosalicylic acid (10 g per 100 ml) followed by centrifugation at 11 600 *g* for 5 min to remove precipitated protein. The supernatant was adjusted to pH 2.0–2.2 with 0.3 *M* sodium hydroxide (approximately 0.2 ml) producing a dilution factor of 1.5. The sample was diluted a further ten-fold with 0.2 *M* lithium citrate diluent buffer (pH 2.2) before analysis.

Deproteinisation of serum samples was performed by first drying 0.1 ml of 12.5% (w/v) sulphosalicylic acid in 95% ethanol in a microfuge tube to which 0.1 ml of serum was then added. The dried residue was mixed with the serum sample and then centrifuged as described for urine to remove the resulting protein precipitate. The serum supernatant was removed and diluted five-fold in 0.2 *M* lithium citrate diluent buffer (pH 2.2) and applied to the analyser.

RESULTS AND DISCUSSION

Construction of a semi-automated amino acid analyser (outlined in Fig. 1) makes use of a laboratory's existing HPLC pumps, injector and detection equipment. The system as shown requires additional equipment consisting of air-tight buffer reservoir bottles, a suitable solenoid switching valve consisting of one out-

let and six inlet ports, a stainles-steel column, a suitable high-efficiency ion-exchange resin which in our case was Dionex DC-4A and a water-tight glass column jacket connected to a suitable water circulating heater bath. The responsiveness of the water heater was 1°C/min. Dimensions of connector tubing are given in Fig. 1. The system shown uses post-column derivatisation of amino acids with OPA–2-ME followed by fluorescence detection.

An alternative system which we have found equally effective is the use of post-column derivatisation of amino acids with ninhydrin which requires a fixed- or variable-wavelength LC spectrophotometer capable of detection at or around 570 or 440 nm. For detection of amino acids with ninhydrin a reaction coil consisting of stainless-steel tubing (25 m×0.3 mm) immersed in a suitable oil bath (Chemlab Instruments) and kept at a temperature of 100°C was utilized. This gave a dwell time of approximately 6 min when using a combined flow-rate from the buffer pump and ninhydrin reagent pump of 0.3 ml/min. An additional 2 m of tubing (0.3 mm I.D.) was connected to the detector outlet which acted as a bubble suppressor. The detector used in our case was an LC Waters M-440 (dual channel) fitted with 436- and 546-nm filters.

A BBC Model B microcomputer was chosen as an intelligent controller for the apparatus. This computer was chosen because of its versatility and common usage in manufacturing and research laboratories in the U.K. and Europe. The computer is an 8-bit 6502 based machine with an on board 6522 VIA (versatile interface adapter). The operating system (BBC operating system) calls allow access

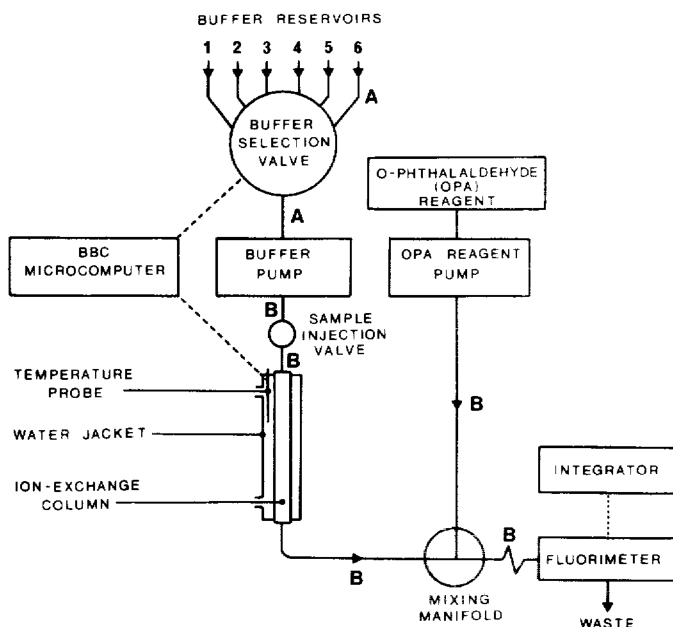

Fig. 1. Schematic diagram showing the single-column amino acid analyser system described in the text. Sites under computer control are indicated by dashed lines. Tubing internal diameters are indicated as follows: A, 1.5 mm; B, 0.3 mm. Stainless-steel tubing was used on the outlets of both pumps up to the mixing manifold. All other tubing was made of PTFE.

to this, as an on board 12-bit analogue-to-digital converter and an elapsed time clock. The latter two are also accessible to Basic users via Basic commands. The Basic interpreter is relatively fast and allows the use of user-defined procedures and function. The Basic program monitored column temperature and the clock of the computer. In response to these inputs the temperature and solvent selection valve were controlled.

Column temperature was monitored via a thermistor probe inserted into the glass heating jacket surrounding the column and connected to the analog port of the computer. This thermistor formed half of a potential-divider connected between the reference voltage output and ground, its junction with a fixed resistor being connected to analog channel 1. The advantage of this simple system is that the measured temperature is essentially unaffected by reference voltage drift. The heater control algorithm has a 0.2°C dead zone to reduce the relay activity. The heater and solvent selection switches were both driven from the computer's user port via an interface unit (Fig. 2).

A simplified flow chart for the control program is shown in Fig. 3. When entered, the program sets up a number of initial conditions, e.g. side B of the user VIA is set for output and the temperature probe calibration data is read into an array. From this point the program is controlled from a menu page. Run conditions can be set up from the keyboard, loaded from or saved to disk. The solvent selection switch can be incremented manually or a run can be started. Finally, exit from the program can be with an orderly return to normal. The program can support six temperature changes and six solvents. Before a run, the program enters an indefinite loop with a single controlled temperature for the purpose of equilibrating the column in solvent number 1. The program returns to the equilibration loop after each run. A "key press" starts the run conditions by moving control to a second loop, during which the column temperature follows a preprogrammed sequence and the solvent can be changed at pre-set intervals. A plot on the computer VDU of temperature versus time (with solvent changes indicated) provides a visual check on the progress of the run.

Elution conditions optimised for separation of components in complex mixtures such as physiological fluids (shown in Table I) were found to be adequate for continuous analyses every 5 h, including column equilibration time. Analysis of a mixture of physiological standards (Fig. 4) shows good separation of nearly all amino acids enabling quantitative analysis of over thirty components. Proline, which is not shown in Fig. 4, was found to elute just prior to glycine and may be detected by post-column reaction with ninhydrin and spectrophotometric detection at 436 nm.

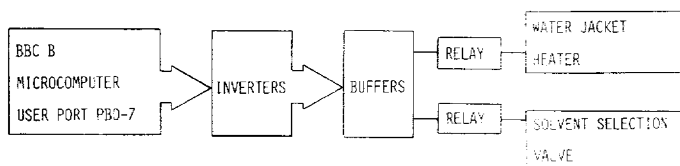

Fig. 2. Schematic diagram of the interface unit which allows the BBC microcomputer to control the water jacket heater and solvent selection valve.

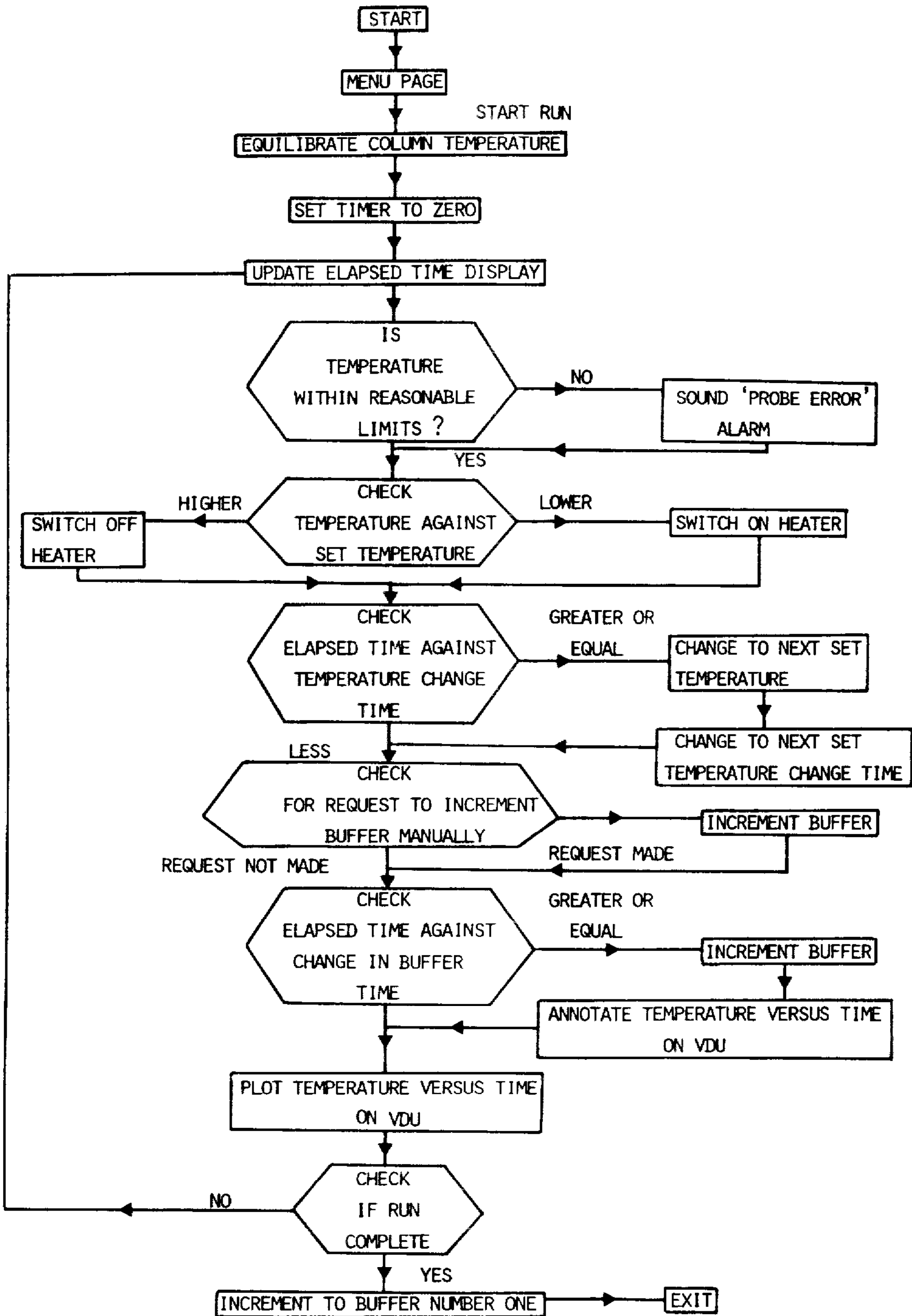

Fig. 3. Flow diagram demonstrating how the computer software allows selection and subsequent control of sequential elution conditions of the analyser system.

TABLE I

ELUTION CONDITIONS PROGRAMMED INTO THE BBC COMPUTER FOR SEPARATION OF AMINO ACID COMPONENTS PRESENT IN PHYSIOLOGICAL FLUIDS

Picobuffer	pH	Elution time (min)	Column temperature (°C)
1	2.0	38	40
2	2.08	30	40
3	2.1	22	40
3	2.1	16	60
4	2.25	38	60
5	2.25	68	60
Lithium hydroxide (0.3 *M*)	12.5	30	60

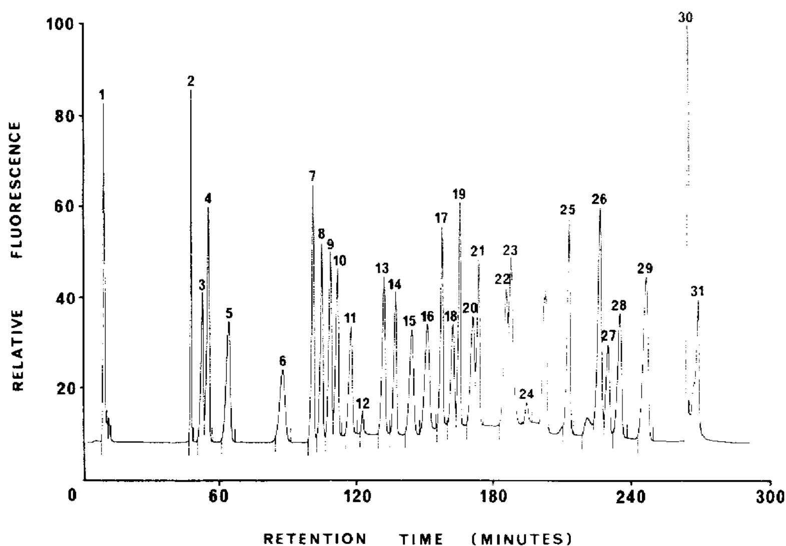

Fig. 4. Chromatogram showing typical separation of a standard physiological mixture containing 10 nmol of each component in an injection volume of 100 μl. Elution conditions were those shown in Table I. Amino acid components were as follows: 1 = taurine; 2 = aspartate; 3 = threonine; 4 = serine; 5 = glutamate; 6 = α-aminoadipic acid; 7 = glycine; 8 = alanine; 9 = citrulline; 10 = γ-amino-N-butyrate; 11 = valine; 12 = cystine; 13 = methionine; 14 = L-cystathionine; 15 = isoleucine; 16 = leucine; 17 = norleucine; 18 = tyrosine; 19 = not determined; 20 = phenylalanine; 21 = β-alanine; 22 = DL-γ-amino-isobutyrate; 23 = γ-amino-N-butyrate; 24 = ammonia; 25 = ornithine; 26 = lysine; 27 = 1-methylhistidine; 28 = histidine; 29 = 3-methylhistidine; 30 = tryptophan; 31 = arginine; 32 = phosphoserine; 33 = glutamine.

Assessment of reproducibilities of column retention times and peak areas (Table II) was performed by applying a series of physiological standards to the column. The coefficients of variation (C.V.) for retention times ranged from 0.3 to 4.0%, the majority of components having a C.V. of about 2.0%. Peak areas were more variable, the majority producing a C.V. of between 1 and 5%. Components

TABLE II

REPRODUCIBILITY OF RETENTION TIMES AND PEAK AREAS FOR SEPARATION OF AMINO ACIDS

The data represent the mean values from five separate experiments. Elution conditions were those described in Table I. Norleucine was used as an internal standard for calculation of peak areas.

Amino acid component	Retention time		Peak area
	Mean (min)	C.V. (%)	C.V. (%)
Phosphoserine	6.8	3.9	0.7
Taurine	11.3	2.0	1.4
Phosphoethanolamine	13.6	2.1	1.8
Aspartic acid	39.0	2.2	6.0
Threonine	51.5	1.9	5.5
Serine	54.7	1.7	5.2
Glutamic acid	59.8	0.6	4.9
α-Aminoadipic acid	88.4	3.7	7.3
Glycine	100.0	1.3	6.5
Alanine	103.6	2.6	4.8
Citrulline	106.3	2.3	2.8
Valine	116.5	2.8	4.8
Cystine	120.9	2.3	3.7
Methionine	129.7	2.4	2.3
Isoleucine	142.5	2.7	2.0
Leucine	148.7	2.4	3.3
Norleucine	153.9	1.7	-
Tyrosine	158.4	1.7	3.8
β-Alanine	168.1	1.6	2.3
Phenylalanine	170.1	1.9	3.3
Ammonia	192.9	1.0	6.7
Hydroxylysine	198.4	1.6	4.8
Ornithine	208.9	1.7	4.5
Lysine	222.5	1.8	3.2
1-Methylhistidine	227.5	1.8	5.6
Histidine	232.7	1.9	3.2
3-Methylhistidine	244.6	1.8	4.1
Tryptophan	253.9	0.29	4.9
Carnosine	255.2	0.47	1.3
Arginine	258.6	0.46	3.8

giving a notably high C.V. were α-aminoadipic acid and ammonia. Amino acid standards were used to assess the correlation between peak-area ratio and concentration. A chosen concentration range of between 1 and 12.5 nmol of each amino acid per 100-μl injection produced a linear relationship to peak-area ratio, for all amino acids tested. The quantitative detection limit of the system was 10 pmol, below which baseline noise interferes with poorly fluorescent derivatives. These results are consistent with other reports which have also demonstrated accurate detection down to these levels [14,15].

Chromatograms shown in Fig. 5A and B demonstrate the application of the

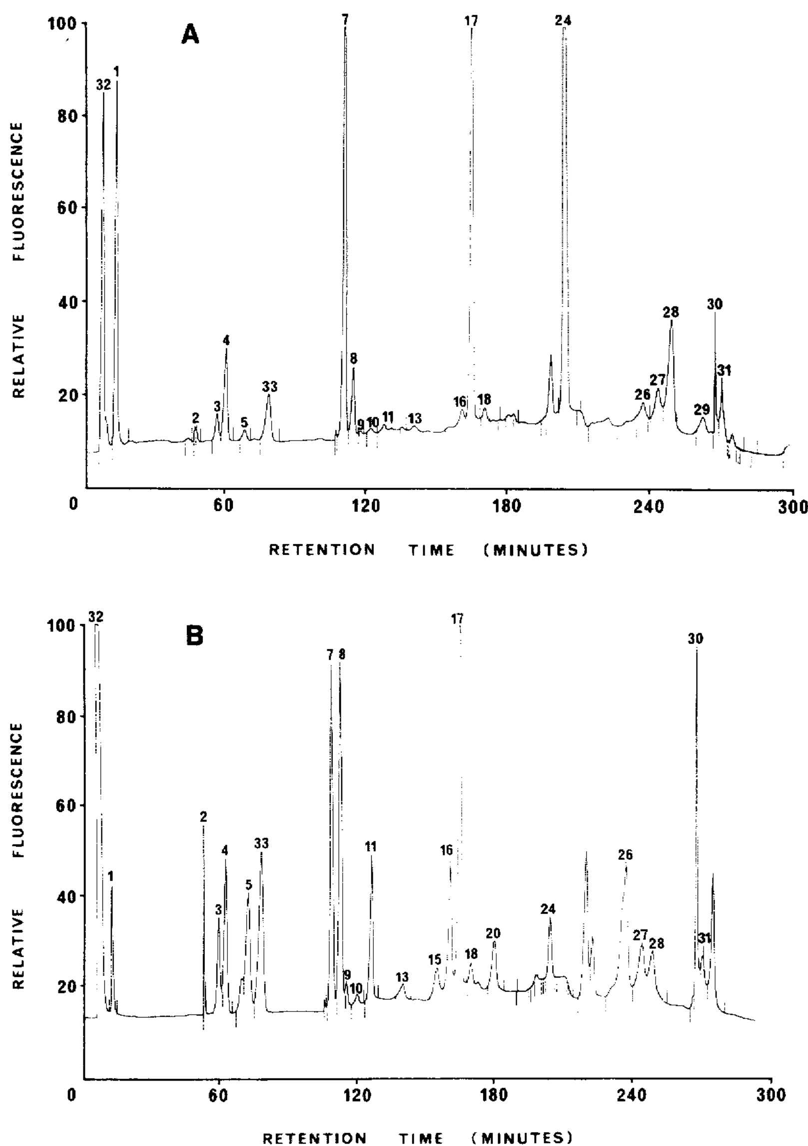

Fig. 5. Chromatogram showing separation of (A) normal human urine sample and (B) normal human serum sample. Fluorimeter sensitivity, injection volume and peak definitions are the same as those defined in the legend to Fig. 3. Urine amino acid levels ranged from 14 pmol (citrulline, peak 9) to 57 nmol (ammonia, peak 24).

system to analysis of human urine and serum, respectively. Comparison of the serum amino acid levels calculated from Fig. 5B with those quoted in the literature (Table III) indicate good comparability thus demonstrating the reliability of this sytem. Only glutamine was found to be present at lower levels than those quoted which may be explained by the inherent instability of this amino acid in stored plasma [16]. A prominant ammonia peak found in urine is the most striking difference between the two biological fluids. However, ammonia determina-

TABLE III

ASSESSMENT OF MEASUREMENT RELIABILITY

Comparison of serum amino acid levels calculated from Fig. 5B with those previously reported. Data after Ibbott [17].

Amino acid component	Calculated serum values (μM)	Normal serum ranges (μM)
Taurine	51	35–140
Aspartic acid	42	11– 54
Threonine	78	75–250
Serine	90	61–190
Glutamic acid	108	0–120
Glycine	240	130–490
Alanine	246	170–500
Citrulline	15	12– 55
γ-Amino-N-butyric acid	8	8– 35
Valine	123	120–330
Methionine	36	13– 39
Isoleucine	36	35–100
Leucine	72	69–160
Tyrosine	60	32– 87
Phenylalanine	63	34–120
Lysine	150	90–260
Histidine	60	56–120
Arginine	49	46–150
Glutamine	310	420–760

tion can only be assessed on a semi-quantitative basis in the present system due to buffer contamination which is evident in Fig. 5A where a broad raised plateau surrounds the sharper sample peak (peak 24). Incorporation of a suitable 'ammonia trap' in the system would overcome problems of contamination and allow quantitative determination of ammonia and compounds with a similar retention time if so required. A consistent feature of urine and plasma samples stored frozen for a period of time is a large initial peak in the phosphoserine position. This may be a degradative product or an artifact produced during sample processing although this peak was not present in standard amino acid mixtures which underwent identical processing.

The system developed here is ideally suited to clinical and research laboratories whose sample turnover does not merit purchase of a ready-made fully automated ion-exchange analyser but who already possess HPLC equipment. Post-column derivatisation with OPA–2-ME reagent offers ease of derivatisation, increased sensitivity and selectivity of component detection compared to other methods. The main disadvantage of a fluorimetric detection system is that only primary amines can be analysed so that components such as proline, hydroxyproline and urea remain undetected. Proline and hydroxyproline may be detected with OPA–

2-ME if the column eluent is reacted with hypochlorite [14] prior to derivatisation, although this would necessitate the use of a further pump. However, this system is equally adaptable to post-column derivatisation with ninhydrin if detection of these components is required.

In conclusion, the ion-exchange system for the analysis of amino acids which has been described can be simply and economically constructed for use with existing HPLC equipment. The versatility of the system is such that it will facilitate the rapid alteration of elution conditions for the analyses of complex mixtures or specific components which are presently difficult to analyse using existing reversed-phase HPLC methods.

NOTE

The program for the computer software described in this report may be obtained on request.

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