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Measurement of plasma and urine amino acids by high-performance liquid chromatography with electrochemical detection using phenylisothiocyanate derivatization

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SUMMARY

The use of reversed-phase liquid chromatography (LC) with pre-column derivatization for the analysis of amino acid mixtures is becoming established as a possible cheaper alternative to commercial amino acid analysers. The available derivatization procedures all have disadvantages when applied to clinical samples, partly due to the interferences found with body fluids when ultraviolet or fluorescence detection is used. An LC method is described for the separation of amino acids in blood or urine, using pre-column derivatization with phenylisothiocyanate (PITC), gradient elution and electrochemical detection. The use of electrochemical detection of PITC derivatives virtually eliminates interferences and enables the secondary amino acids to be measured. Examples are shown of normal urine and plasma and samples from patients with cystinuria and maple syrup urine disease.

INTRODUCTION

The determination of amino acids in clinical chemistry is of particular importance in the diagnosis and monitoring of the inherited disorders of amino acid metabolism and is also of value in many nutritional studies. Amino acids are usually measured using chromatographic techniques such as thin-layer

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chromatography (TLC), ion-exchange chromatography and high-performance liquid chromatography (LC). TLC is at best semi-quantitative while ion-exchange chromatography is the method on which most commercially available amino acid analysers are based. The substantial growth in the applications of LC in clinical chemistry over the past decade has resulted in many hospital laboratories obtaining LC equipment. The use of LC could therefore provide a relatively simple and inexpensive system for amino acid analysis in the routine or research laboratory.

A number of derivatization procedures have been reported to give sufficient sensitivity and adequate chromatographic properties for measurement of amino acids by LC, using both pre- and post-column derivatization: dansyl chloride (pre-column [1]), dabsyl chloride (pre-column [2]), phenylisothiocyanate (PITC) (pre-column [3,4]), 9-fluorenylmethyl chloroformate (pre-column [5]), *o*-phthalaldehyde (OPA) (pre- or post-column [6]) and ninhydrin (post-column [7]).

The samples analysed in the clinical laboratory are usually blood (serum or plasma) and urine. Some of the above mentioned derivatives have particular disadvantages with these sample media, are unstable or require long or complex derivatization procedures.

Dansyl or dabsyl chloride give poor yields unless present in considerable excess, in which case fluorescent dansyl sulphonic acid compounds can be produced. The use of OPA or 9-fluorenylmethyl chloroformate normally requires fluorescence detection which is complicated by the presence of a large number of compounds in blood and urine with native fluorescence. During the reaction step the 9-fluorenylmethyl chloroformate forms fluorenylmethyl alcohol which must be removed by extraction (solid-phase or liquid–liquid) to prevent interference. The use of electrochemical detection with OPA derivatives has been described by several groups [8,9] which overcomes the problems inherent with the use of fluorescence. OPA derivatives, however, have half-lives of 10–60 min and the imino acids do not form derivatives unless pre-treated with sodium hypochlorite. Ninhydrin gives products with an absorption maximum at 580 nm for primary amines and at 440 nm for secondary amines, requiring a switchable-wavelength detector to measure both. The limit of detection of ninhydrin derivatization is low (nmol) compared with the other derivatives (pmol) and post-column derivatization requires a second pump to introduce the reagent.

In their original paper describing the use of PITC for measurement of amino acids Heinrikson and Meredith [4] showed that the derivatives were stable and gave good chromatographic separation using reversed-phase LC. Detection of the phenylthiocarbamyl (PTC) amino acid derivatives can be achieved using their absorption at 254 nm, but at this wavelength both unreacted PITC and other compounds present in blood or urine can interfere [3]. The amino acid PTC derivatives have been reported to be electrochemically active [10]

suggesting that electrochemical detection may be a viable alternative to UV absorption. This paper describes the measurement of amino acids in human blood and urine samples using reversed-phase LC, after PITC derivatization, with electrochemical detection.

EXPERIMENTAL

Materials and reagents

Mixed amino acid standards of three types, seventeen amino acids, acids and neutrals and basics were obtained from Sigma (Poole, U.K.). Individual amino acids, sodium acetate trihydrate, PITC and triethylamine were obtained from BDH (Poole, U.K.). Solvents, acetonitrile (HPLC grade), methanol, dichloromethane and absolute alcohol (Analar grade) were purchased from Fisons (Crawley, U.K.).

The PITC reagent and the coupling solvent were made fresh each day. PITC (50 μl) was added to methanol (350 μl). The coupling solvent was prepared by adding triethylamine (50 μl) to methanol (950 μl).

Standard preparation

The mixed amino acid standards were used without further preparation. Individual standards were prepared (1 g/l) in 0.1 *M* hydrochloric acid. Norleucine was used as internal standard. To prepare the standards for derivatization 10 μl of an individual or a mixed amino acid standard was added to 10 μl of internal standard.

Sample preparation

Serum and urine collected without preservative were stored frozen until assayed. Samples were deproteinized with absolute alcohol prior to derivatization. Internal standard (10 μl) was added to $50/x$ μl of urine (where x=creatinine in mmol/l). Absolute alcohol (200 μl) was added, mixed and then centrifuged. The supernatant was then taken off into a clean tube and evaporated until dry using a rotary evaporator. The urine was then ready for derivatization. For the serum samples 400 μl of absolute alcohol were added to 100 μl of serum and the procedure for urine was then followed. If necessary a further protein precipitation step could be carried out for the serum samples.

Derivatization

Amino acids in standards or deproteinised urine and serum were reacted with PITC in the presence of a coupling solvent to yield the PTC derivatives as shown in Fig. 1.

To the standard, dried plasma/serum or urine samples, coupling solvent (200 μl) and PITC (10 μl) were added. This was mixed and allowed to react for 5 min before evaporation using a rotary evaporator. Buffer A (500 μl) was added

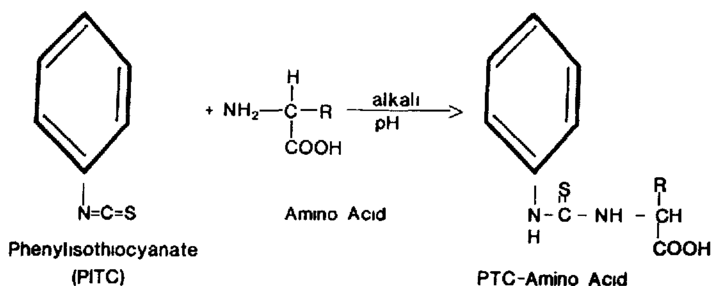

Fig. 1. Formation of the phenylisothiocyanate amino acid derivatives.

to the dried residue and when redissolved, dichloromethane (200 μl) was added. This was mixed for 1 min on a vortex-mixer and centrifuged at 1200 *g* for 1–3 min. An aliquot (100 μl) of the aqueous layer was taken off for chromatography.

Chromatography

Two buffers were prepared for chromatography. Buffer A was sodium acetate trihydrate (1.36 g) adjusted to pH 6.40 (±0.01) with orthophosphoric acid (2%) and made up to 1 l with deionized water. Buffer B was sodium acetate trihydrate (1.36 g) adjusted to pH 6.40 (±0.01) with orthophosphoric acid (2%), made up to 400 ml with deionised water to which acetonitrile (600 ml) was added. The buffers were filtered before use and then degassed by bubbling helium through each during use.

A Spectra-Physics SP8700 XR gradient HPLC system including a Rheodyne 7125 injection valve (20-μl loop) (Spectra-Physics, St. Albans, U.K.) was used. The column was a Hypersil-ODS cartridge column (250 mm×4.6 mm I.D., 5 μm), (Jones Chromatography, Hengoed, U.K.). Either an LCA 15 or CHROMAJET electrochemical detector (EDT Research, London, U.K.) was used and the output was monitored with a Spectra-Physics CHROMJET computing integrator (Spectra-Physics).

LC gradient elution

The solvent gradient programmed into the solvent delivery system is shown in Table I and graphically in Fig. 2. This was optimised over a period of time.

Electrochemical detection

The detector settings were: operating potential, +1.10 V (glassy carbon electrode versus Ag/AgCl reference electrode); sensitivity, 3 μA (LCA-15) or 10 μA (CHROMAJET); time constant, 1 s.

TABLE I

SETTINGS FOR THE GRADIENT ELUTION PROGRAMME FOR SEPARATION OF THE MOST COMMONLY ENCOUNTERED AMINO ACIDS

Time (min)	Buffer A (%)	Buffer B (%)	Flow-rate (ml/min)
0	100	0	1.0
20	87	13	1.0
65	45	55	1.0
67.5	0	100	2.0
70	0	100	2.0
75	100	0	2.0
80	100	0	2.0

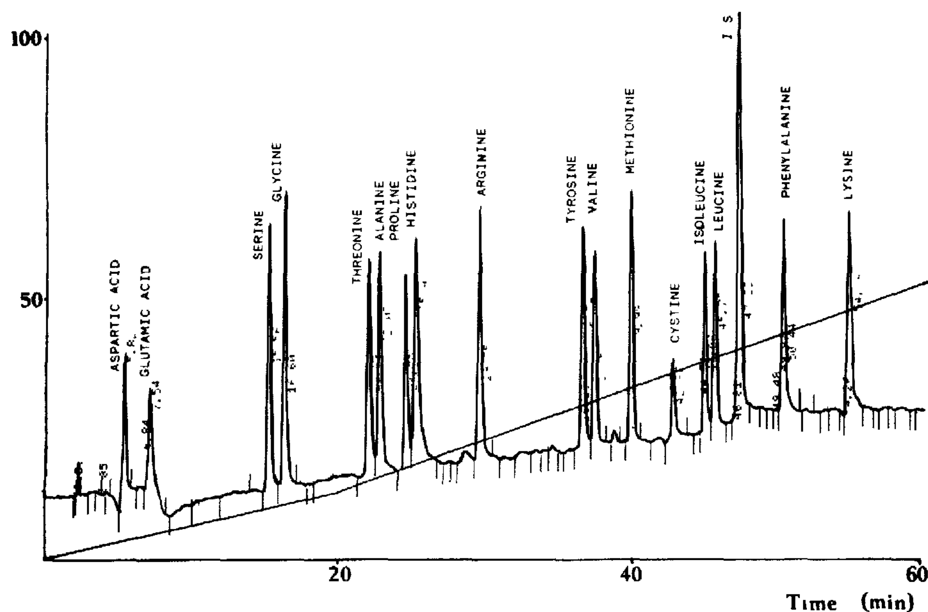

Fig. 2. Separation by gradient elution of the seventeen amino acid standard (Sigma). The change in the buffer composition is shown as the percentage of buffer B. Internal standard (IS) is norleucine.

RESULTS

Resolution of standard amino acid mixtures

The seventeen amino acid standard (Sigma) was derivatized using the procedure described. The separation of the amino acids is shown in Fig. 2. All of

the amino acids in the standard were clearly resolved. A mixture of available amino acid standards was prepared to give a standard containing forty amino acids (including the internal standard). The retention times and the relative retention times are shown in Table II. Most of the amino acids are resolved with the gradient chosen. There are some peaks that are very close together, e.g. glycine/asparagine, methionine/cystathionine and phenylalanine/hydroxylysine but the relative concentrations of each pairing in typical urine or blood samples mean that there are few clinical conditions where mis-identification might occur (possibly cystathioninuria). Occasionally differences were seen in the resolution usually involving proline co-eluting with another amino acid. This was found to be due to proline being sensitive to small pH differences in the buffers. The chromatograms were otherwise reasonably consistent.

Stability of the derivatives

The derivatives appear stable for long periods if stored dry at $-20\,^{\circ}C$ (no significant degradation was seen after two months). There is some loss if the derivatives are stored in buffer at room temperature. This loss is variable for individual amino acids and can be minimised (5–10% over seven days) if the derivatives are kept at $4\,^{\circ}C$.

Linearity of quantitation

The amino acids can be quantitated with this method using measurement of peak areas relative to the internal standard. The linearity varies for each amino acid and has not been assessed for all those shown in Table II. For example, glycine is linear in the range 0–2000 μmol/l, the major limitation to linearity being that higher concentrations went off-scale on the detector (the CHROMAJET was better in this respect having a 10-μA upper scale rather than the 3 μA of the LCA 15).

Selection of operating potential

There is considerable variability in the optimum operating potential for the individual amino acids. A potential of +1.1 V was chosen to give the best limits of detection for all the amino acids. Cystine and homocystine particularly require the high voltage to allow physiological concentrations to be determined. The dichloromethane extraction step removes many potential interferents as well as excess unreacted PITC.

Sample chromatograms

Chromatograms obtained from a normal urine and a normal serum are shown in Fig. 3A and B, respectively. Fig. 4 shows chromatograms obtained from urine from a patient with cystinuria (A) and plasma from a patient with maple syrup urine disease (B).

TABLE II

MEAN RETENTION TIMES AND RELATIVE RETENTION TIMES (RELATIVE TO NORLEUCINE) FOR FORTY AMINO ACIDS LIKELY TO BE FOUND IN BODY FLUIDS

Amino acid	Mean retention time (*min*)	Mean relative *retention time*
1 Phosphoserine	3.82	0.084
2. Aspartic acid	5.44	0.120
3. Glutamic acid	6.83	0.150
4. γ-Aminoadipic acid	10.35	0.229
5. Hydroxyproline	12.26	0.271
6. Phosphoethanolamine	12.45	0.276
7. Serine	14.52	0.321
8. Glycine	15.52	0.344
9 Asparagine	15.54	0.345
10. Sarcosine	16.91	0.375
11. β-Alanine	17.42	0.386
12. Taurine	18.98	0.421
13. γ-Aminobutyric acid	20.48	0 454
14. Citrulline	20.63	0.458
15. Threonine	21.11	0.468
16. Alanine	21.75	0.482
17. β-Aminoisobutyric acid	22.27	0.494
18. Proline	23.48	0.521
19. Histidine	23.91	0.530
20 Carnosine	25.71	0.570
21. Arginine	28.22	0.626
22. 1-Methyl histidine	28.39	0.630
23. 3-Methyl histidine	28.39	0.630
24. α-Aminobutyric acid	28.60	0.634
25. Anserine	29.06	0.644
26. Tyrosine	35.02	0.777
27. Valine	35.76	0.794
28. Ethanolamine	37.65	0.835
29. Methionine	38.10	0.845
30. Cystathionine	38.19	0.847
31. Cystine	40.88	0.907
32. Isoleucine	42.80	0.950
33. Leucine	43.47	0.965
34. Norleucine	45.07	-
35. Hydroxylysine 1	47.78	1.060
36. Phenylalanine	48.20	1.069
37. Hydroxylysine 2	48.46	1.075
38. Ornithine	49.67	1.102
39. Tryptophan	50.16	1.112
40. Lysine	52.79	1.171

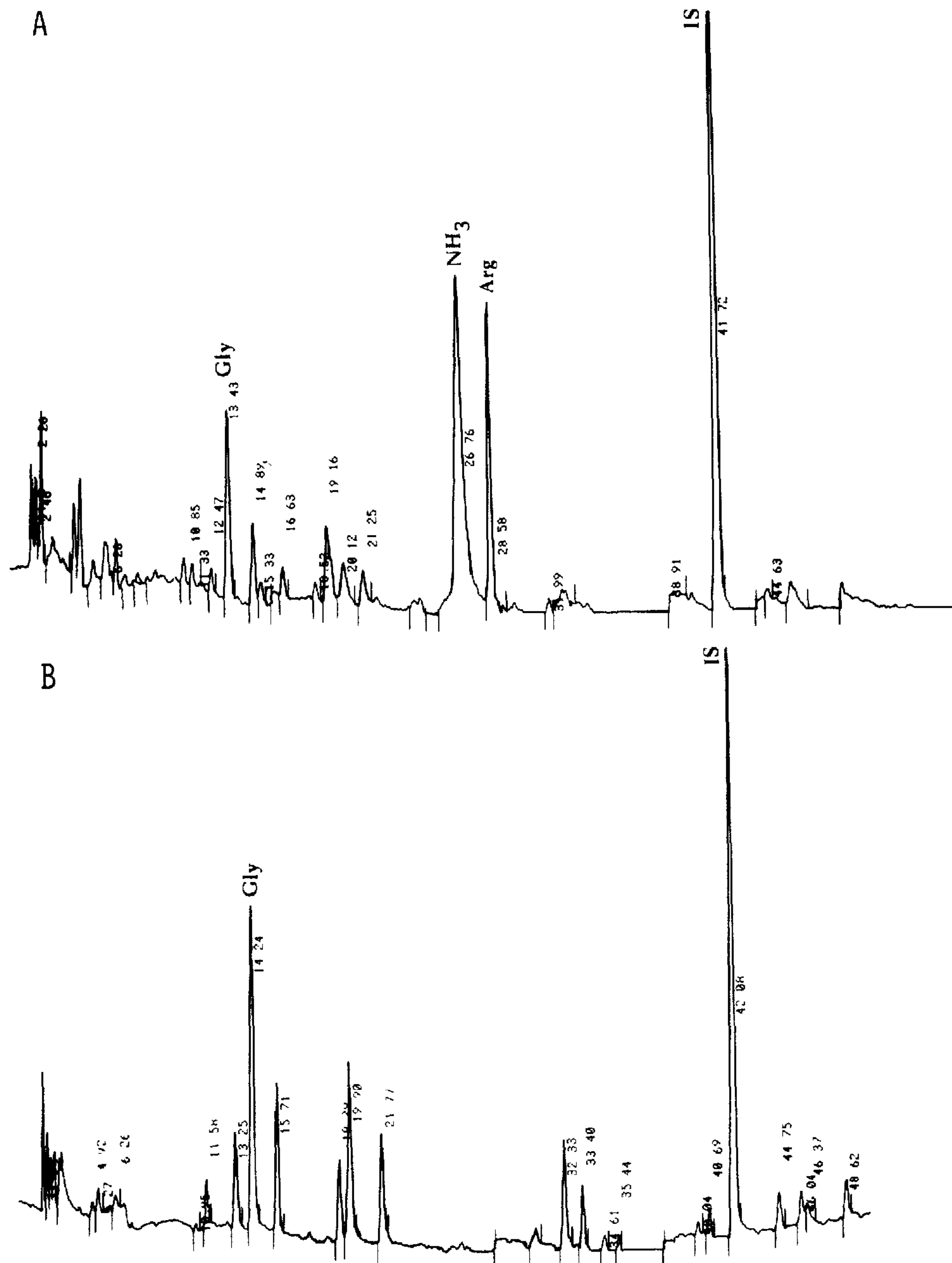

Fig. 3. (A) Chromatogram of the amino acid separation of a normal urine sample. Peaks: Gly = glycine; Arg = arginine; NH_3 = ammonia; IS = internal standard. (B) Chromatogram of the amino acid separation of a normal serum sample Peaks Gly = glycine; IS = internal standard.

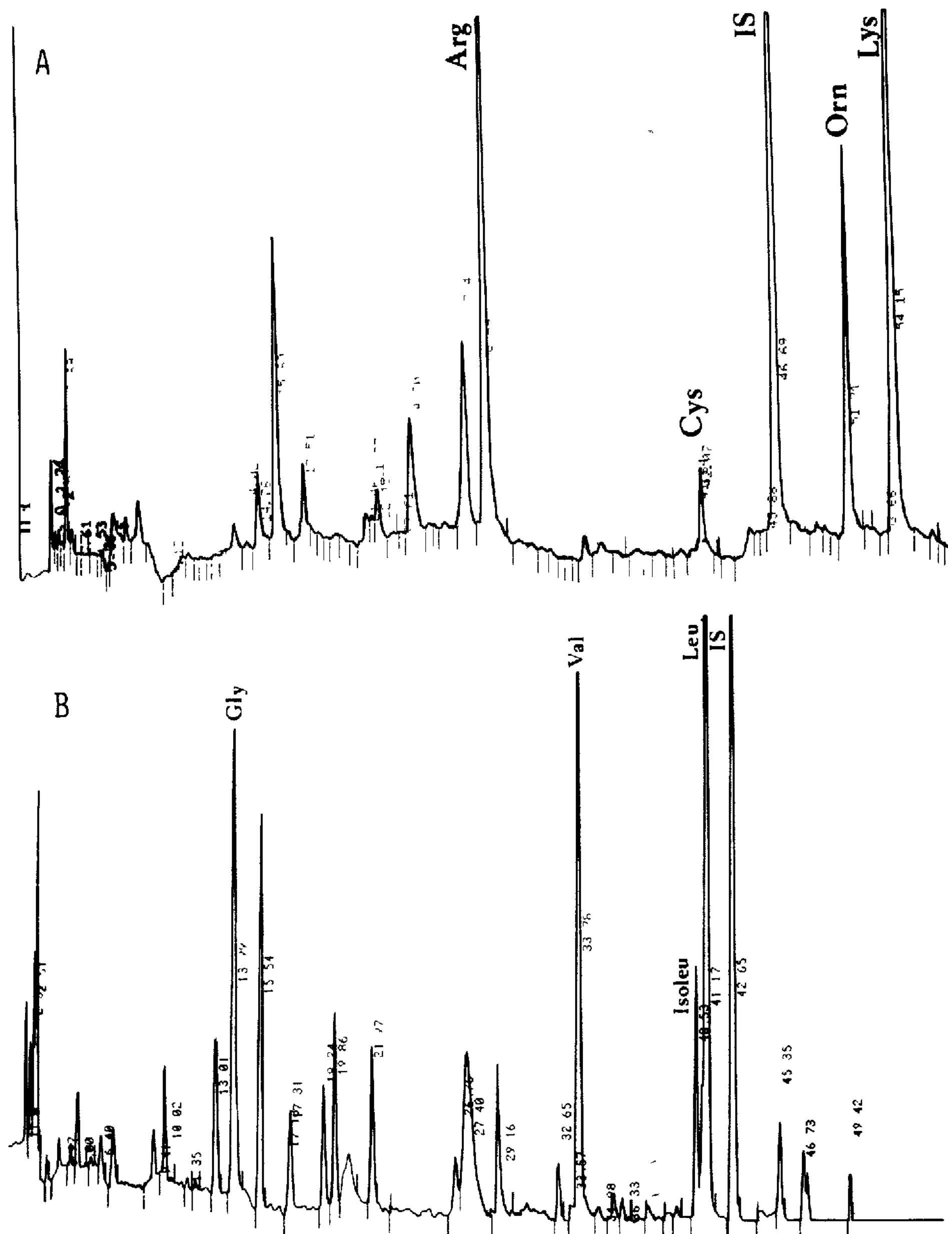

Fig. 4. (A) Chromatogram of the amino acid pattern of a urine sample from a seven-year-old boy with cystinuria. Peaks: Arg = arginine; Cys = cystine; Orn = ornithine; Lys = lysine; IS = internal standard. (B) Chromatogram of the amino acid separation of a plasma sample from a nineteen-year-old girl with maple syrup urine disease. Peaks: Gly = glycine; Isoleu = isoleucine; Leu = leucine; IS = internal standard; Val = valine.

DISCUSSION

The measurement of amino acids in clinical samples has traditionally required dedicated and expensive equipment. The greater availability of LC equipment in clinical laboratories, and in particular the increasing trend towards the use of gradient elution, has resulted in a considerable number of reports of the use of LC for amino acid assay. Ninhydrin and OPA are probably the most commonly used derivatives for this purpose, but both have disadvantages. In particular OPA derivatives are variably stable and are not formed at all by the imino acids. The use of ninhydrin is complicated by the large number of ninhydrin-reacting substances in blood and urine. The use of PITC derivatives for amino acid analysis has been reported many times in the analytical biochemistry literature. In most cases a protein hydrolysate was analysed which allowed the use of UV measurement at 254 nm for detection [11,12]. The unreacted PITC absorbs at this wavelength and has been reported to interfere, producing a large peak in the neutral/basic amino acid region of the chromatogram [12]. Other components of blood and urine may also absorb in this region which might cause difficulties in interpretation of the chromatogram.

The PTC-amino acid derivatives have significant electroactivity at an applied voltage of +1.1 V. This permits the use of electrochemical detection for the amino acids separated by gradient elution as shown in Fig. 2. In serum and urine samples the coarsest scale on the detector was needed to keep the largest peaks (typically glycine and the internal standard in most samples) on-scale. Concentrations of 1–1000 μmol/l can be measured for most of the amino acids so far investigated and further dilution of the derivatized sample maintained linearity to much higher levels. The separation obtained for the most commonly encountered amino acids as shown in Fig. 2 and Table II indicates that the resolution of the system is adequate for most clinical applications and is comparable to that achieved by commercial amino acid analysers using ion-exchange chromatography.

The derivatives formed are stable for long periods of time when stored frozen both dry or in mobile phase. Once reconstituted there is minimal loss of activity for up to seven days at 4°C or three to four days at room temperature. The derivatization process is easy to perform and does not require complex equipment.

A considerable number of samples have now been processed with this system and no interferences have yet been encountered. The only significant non-amino acid peak that has been observed is that of ammonia which runs just after arginine, but does not interfere with its measurement. Other substances, including penicillins, which are known to react with ninhydrin do not interfere with this system. The high applied voltage used would be expected to result in interferences from other electroactive compounds, particularly in urine. The catecholamines and biogenic amines for example are electrochemically active

at this potential but even if they were retained by the gradient their concentrations are 100–1000 fold lower than the PTC amino acid derivatives.

The system described in this paper allows the measurement of amino acids in blood and urine in less than 100 μl of sample with only a simple derivatization step. We believe this is the first description of the use of electrochemical detection for the measurement of PTC amino acid derivatives in clinical samples. The advantage of electrochemical detection is the virtual elimination of the interferences commonly encountered when UV or fluorescence detection is used. The gradient described permits the resolution and identification of the clinically relevant amino acids but can be altered if individual amino acids are being studied. This gives considerable versatility as alanine or glycine, for example, can be measured using a 20–25 min cycle. With commercial amino acid analysers regardless of whether only a single amino acid is wanted a full 80-min run is usually necessary.

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