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RAPID AUTOMATED ION-EXCHANGE ANALYSIS OF PLASMA TYROSINE AND PHENYLALANINE WITH DATA PRINT-OUT

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SUMMARY

A rapid automated method with data print-out is described for quantitation of plasma phenylalanine and tyrosine from 0.100 ml of sample. The system uses the Rank-Hilger Chromaspek amino acid analyser linked to a Digico M16E computer.

Amino acid concentrations up to 3000 μM can be quantitated without repeat dilutions and assessment of precision at the 500 μM level, produced coefficients of variation of 2.2% for tyrosine and 2.5% for phenylalanine. Recovery determinations from a plasma pool gave a mean recovery of 99.4% for tyrosine and 99.7% for phenylalanine.

Correlation with established fluorimetric techniques was excellent (r = 0.986 for tyrosine, r = 0.976 for phenylalanine). By using the same resin column for both the rapid separation of tyrosine and phenylalanine, and the standard physiological fluid separation, full analysis capability is retained with easy interchange between the two systems.

INTRODUCTION

Accurate quantitation of plasma phenylalanine and tyrosine concentrations are essential for diagnosis of phenylketonuria (PKU) and subsequent monitoring of dietary therapy.

Ion-exchange chromatographic techniques for simultaneous assay of both amino acids [1—4] allow accurate sequential estimations from one sample aliquot and offer certain advantages in specificity [3] over the commonly used fluorimetric techniques [5, 6], particularly in determining phenylalanine tyrosine molar ratios, which have been recommended for PKU heterozygote detection [7, 8].

This paper describes a rapid automated analysis for phenylalanine and tyrosine, using the Rank-Hilger Chromaspek amino acid analyser, linked to a Digico M16E computer.

INSTRUMENTATION

The Rank-Hilger Chromaspek amino acid analyser has been described more fully elsewhere [9, 10]. The main elements of the system are outlined below.

Auto sampler

The sampling module consists of a 60-place refrigerated turntable with programmed sampling time adjustable between 5 and 60 sec.

Programmer unit

The programmer unit uses two control devices. A steel drum rotating at constant speed carries a profile map in black tape, which defines the gradient elution system. This is scanned by an electronic reading head which controls mixing of acidic and basic buffers.

There is also a programmer plate which is scanned by a series of photoelectric sensors; this controls column temperature, ninhydrin/wash status, sampling time and computer print out.

Ion-exchange column

7% cross-linked cation-exchange resin of 8 μm nominal bead diameter is contained within a steel column (500 mm × 2.6 mm I.D.) surrounded by an aluminium heating block. By utilising the 500-mm column for the short analysis programme, the full system capability for analysis for complex physiological fluids is retained.

Pumping system

The Chromaspek uses two pump types: a high-pressure Milton-Roy pump delivering buffer to the resin column at a constant flow-rate of 0.150 ml/min (back pressure 3.5 MPa) and a peristaltic pump delivering buffer and aspirated sample to the high-pressure pump and nitrogen and colour reagents to the column outlet.

Colorimetry

The column eluate is mixed with a ninhydrin/cyanide reagent segmented with nitrogen and heated to 95°. The colour development is measured without debubbling by two photometers at 440 nm and 570 nm using fibre optic light guides from a grating monochromator.

Quantitation

Absorbance outputs from the two photometers are displayed on a dual pen potentiometric recorder (Vitatron Series 2001) and input to the interface of Digico Micro 16E computer (8K store of 16 bit words). Each measuring channel is supplied with three amplifier gain settings covering the ranges 0—0.05, 0—0.10, 0—0.2 absorption units. The Chromaspek interface uses an analogue to digital converter, stated to give full scale, at 10% overscale of the instrument range of 0—0.2 absorption. The interface converts analogue information to digital form and enters the digital data to the computer, which then, applies base line correction, determines peak area, relates to internal and

external standard peak areas and at the end of each run, prints out tabulated results in defined concentration units on the associated teletype.

REAGENTS

Acid buffer (pH 2.20)

Citric acid (AR)	10.5 g
1 *M* lithium chloride	150.0 ml
Thiodiglycol (25% v/v in water)	2.5 ml
Brij 35 (10% w/v in 5% methanol)	3.5 ml

Make up to 1000 ml with distilled water.

Basic buffer (pH 11.50)

Citric acid (AR)	10.5 g
Lithium hydroxide monohydrate (AR)	12.6 g
Boric acid (AR)	8.8 g
Ethylenediaminetetraacetic acid (disodium salt)	0.5 g
Brij 35 (10% w/v in 5% methanol)	3.5 ml

Make up to 1000 ml with distilled water.

Ninhydrin (pH 5.50)

Ninhydrin (AR)	10.0 g
Sodium acetate trihydrate (AR)	216.0 g
Glacial acetic acid (AR)	100.0 ml
2-Methoxyethanol	400.0 ml
Brij 35 (10% w/v in 5% methanol)	10.0 ml

Make up to 1000 ml with distilled water.

Wash solution

2-Methoxyethanol	400.0 ml
Brij 35 (10% w/v in 5% methanol)	10.0 ml

Make up to 1000 ml with distilled water.

Sodium cyanide

1% stock solution:

Sodium cyanide (AR)	1.0 g
Sodium carbonate	4.0 g

Make up to 100 ml with distilled water.

A 0.0005% working solution was then made up:

1% stock solution	0.50 ml
4 *M* sodium hydroxide	2.00 ml
Brij 35 (10% w/v in 5% methanol)	10.00 ml

Make up to 1000 ml with distilled water.

Protein precipitant (3% sulphosalicylic acid containing 120 μM norleucine)

Sulphosalicylic acid (AR)	30.0 g
Norleucine (10 m*M* stock solution)	12.0 ml

Make up to 1000 ml with distilled water.

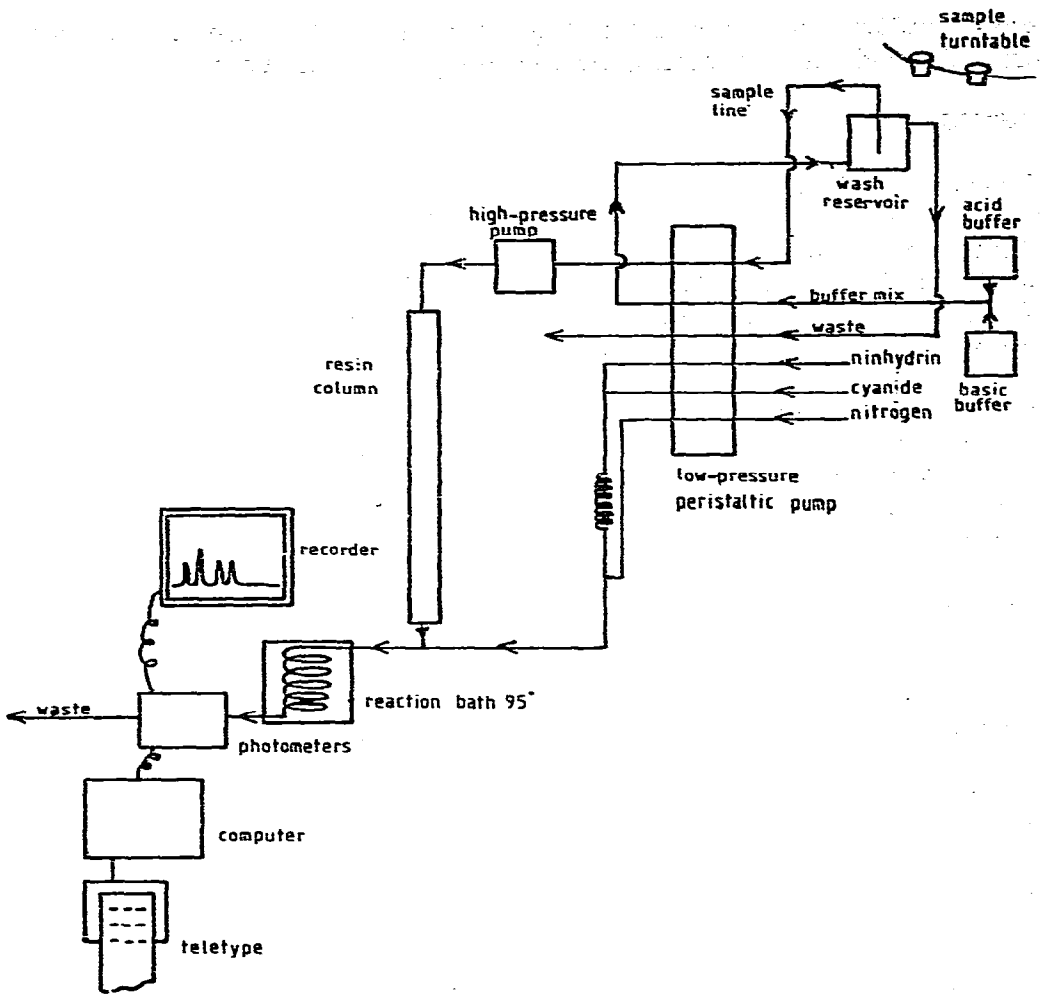

Fig. 1. System format of Rank-Hilger Chromaspek amino acid analyser.

Standard solution

The stock standard was made up of 10 m*M* each of tyrosine, phenylalanine, leucine and isoleucine in 0.025 *M* hydrochloric acid. The working standard comprised of 6.0 ml of the stock standard made up to 100.0 ml with pH 2.20 acid buffer.

SAMPLE PREPARATION

Plasma or working standard (100 µl) was mixed with protein precipitant (500 µl). After 10 min, this was centrifuged at 1500 *g* for 5 min. The supernatant (200 µl) was loaded into a sample cup and placed in position on the sample turntable.

Sample list and internal and external standard concentrations are input to the computer through the teletype. From this point until result print, analysis was totally automatic.

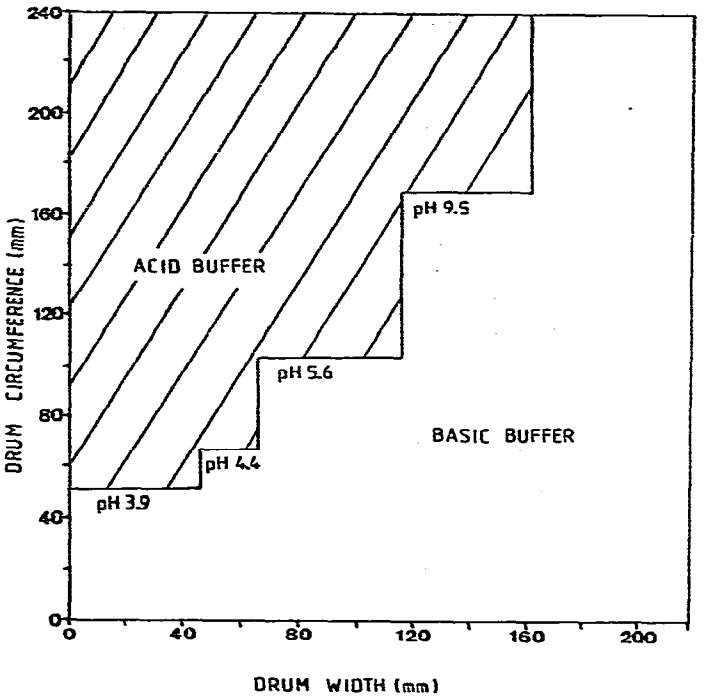

Fig. 2. Programme drum profile producing the pH gradient used in the rapid phenylalanine and tyrosine method.

ANALYSIS SYSTEM

The instrument format is shown in Fig. 1. Analysis conditions were as follows:

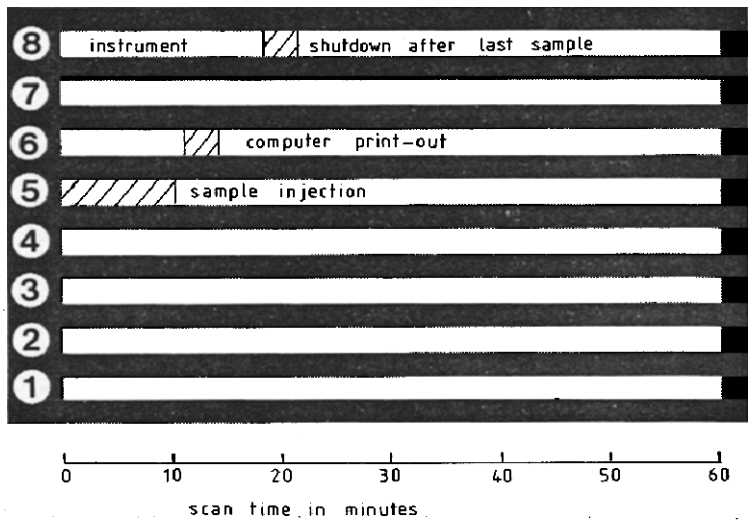

Fig. 3. Programme plate conditions used in the rapid phenylalanine and tyrosine method. Change from masked to clear area initiates function.

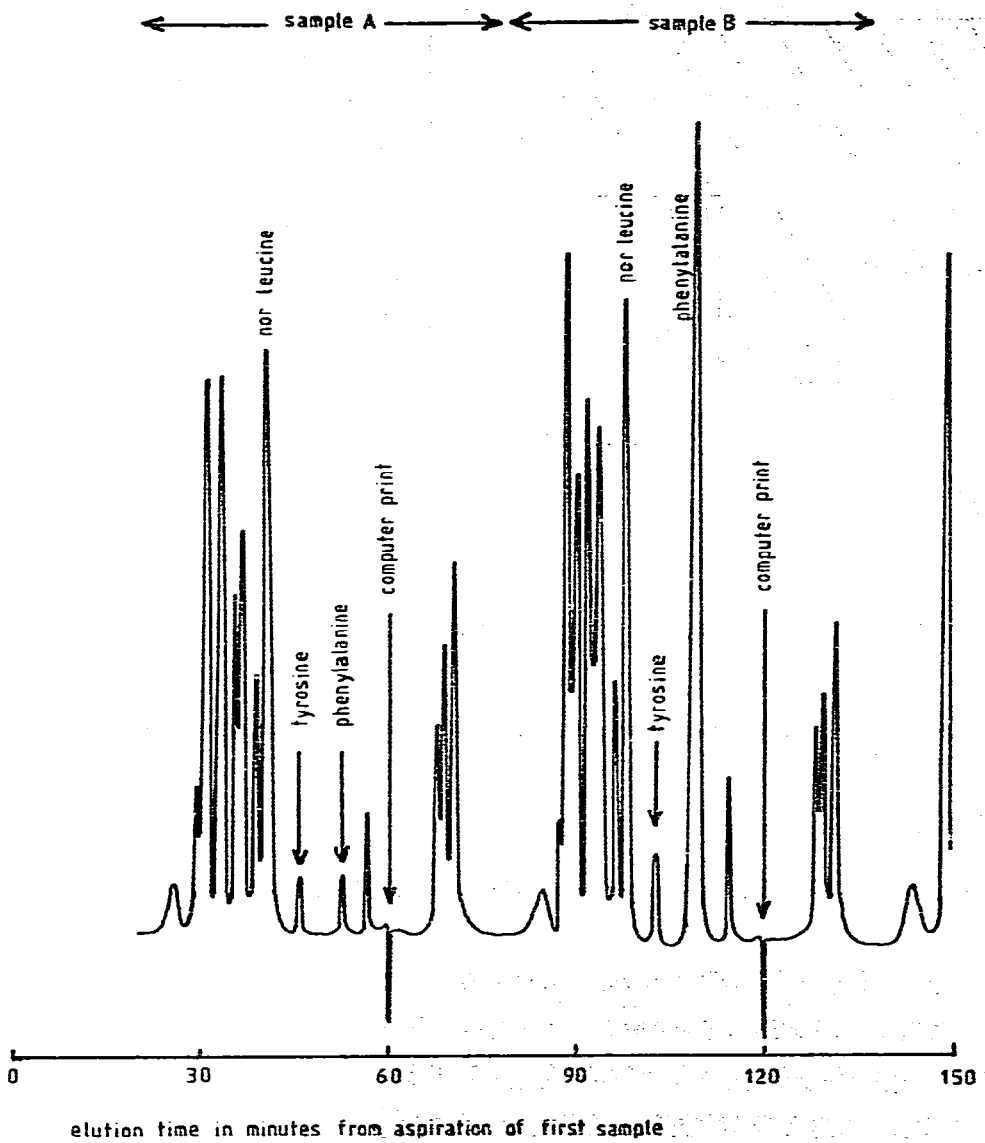

Fig. 4. Typical recorded amino acid profiles for (A) normal plasma sample and (B) PKU plasma sample.

column temperature, 60°; buffer elution rate, 0.150 ml/min; ninhydrin flow-rate, 0.200 ml/min; cyanide flow-rate, 0.050 ml/min; sampling time from analysis commencement, 10 min; total analysis time, 60 min and sample pick up volume (40 sec aspiration) 0.100 ml.

In routine use, one working standard precedes the first sample and every fifth cup contains a similar standard. The buffer profile and programme plate conditions are illustrated in Figs. 2 and 3 and examples of the recorded amino acid profile and computer print-out are shown in Figs. 4 and 5, respectively.

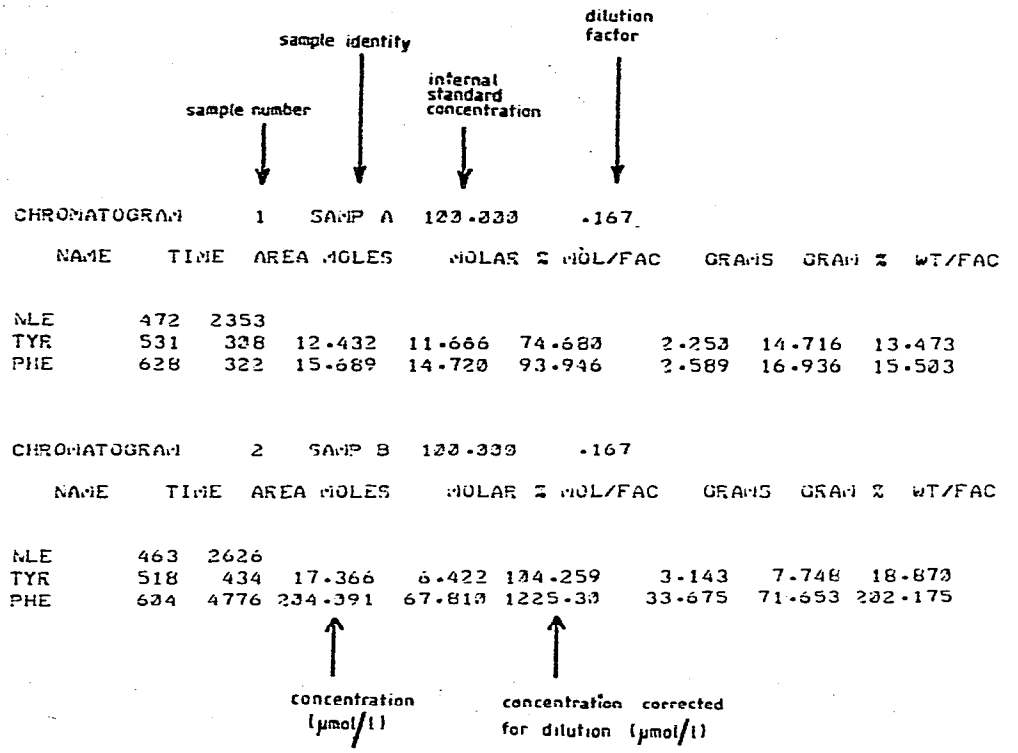

Fig. 5. Computer print-out data relating to profiles shown in Fig. 4. (A) Normal plasma sample and (B) PKU plasma sample.

METHOD ASSESSMENT

Linearity

A series of working standards covering the range 50—4000 *μM* were prepared and analysed by the method, including the sample preparation stage.

Precision

A plasma pool was divided into four samples. Phenylalanine and tyrosine were added to three of these to produce final concentrations covering the range 250—1000 *μM*. All four samples were analysed in replicate (20 assays).

Accuracy

A measure of the accuracy of the method was obtained from an assessment of the percentage recovery of the two amino acids, added to the pooled plasma used in the precision exercise.

In addition a commercially prepared control preparation containing known phenylalanine and tyrosine concentrations, was analysed in replicate and 50 plasma samples were analysed and compared with results obtained using the routinely used fluorimetric techniques [5, 6].

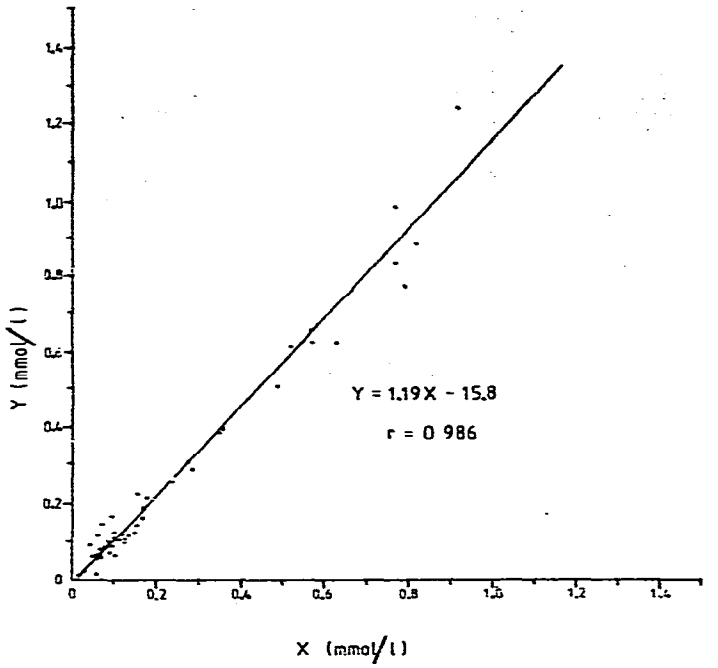

Fig. 6. Correlation between results obtained on 50 plasma sample analyses for tyrosine concentration, using the proposed method (*Y*-axis) and the fluorimetric method (*X*-axis).

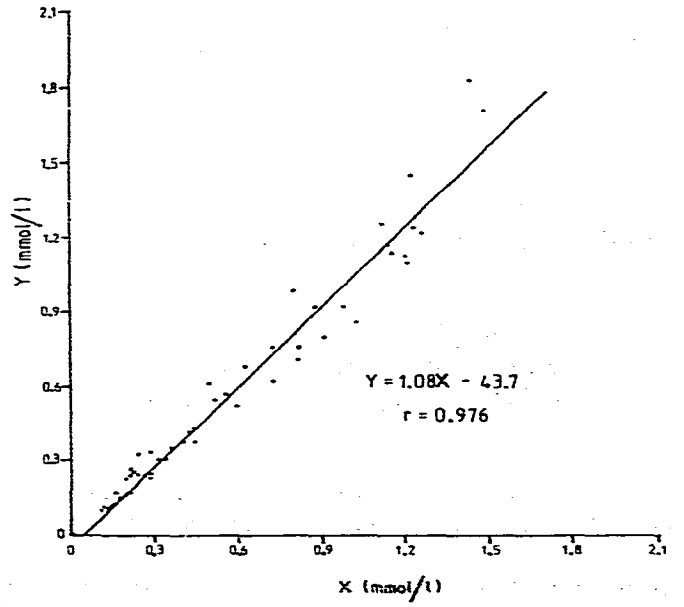

Fig. 7. Correlation between results obtained on 50 plasma sample analyses for phenylalanine concentration using the proposed method (*Y*-axis) and the fluorimetric method (*X*-axis).

RESULTS

Linearity

Using a recorder response range of 0—0.10 absorption units, i.e. mid gain

setting, it was found that although full-scale deflection on the recorder profile occurred at amino acid concentrations of about 1400 μM, computer response as printed sample concentrations was linear for both tyrosine and phenylalanine up to concentrations of 3000 μM.

Precision

Four pooled plasma samples containing varying concentrations of phenylalanine and tyrosine were analysed in replicate (20 assays). Mean phenylalanine concentrations were 79, 308, 532 and 984 μM, respectively, with corresponding coefficients of variation of 3.26%, 2.97%, 2.50% and 2.10%.

For the same samples, mean tyrosine concentrations were 81, 309, 536 and 987 μM, respectively, with corresponding coefficients of variation of 3.02%, 2.71%, 2.23% and 1.92%.

Accuracy

Recovery of phenylalanine and tyrosine added to a plasma pool containing 79 μM of phenylalanine and 81 μM of tyrosine, was determined at three concentration levels. For phenylalanine, percentage recovery (20 replicates) was 99.69 ± 2.94% at a concentration of 309 μM, 99.77 ± 2.51% at a concentration of 534 μM and 99.62 ± 2.10% at a concentration of 988 μM.

Corresponding figures for tyrosine were, 99.20 ± 2.71% at a concentration of 311 μM, 99.95 ± 2.23% at a concentration of 536 μM and 99.64 ± 1.92% at a concentration of 991 μM.

Analysis (20 replicates) of a Sigma Metabolite Control Type I (product No. S 3005), gave a mean phenylalanine concentration of 244.0 ± 7.9 μM and a mean tyrosine concentration of 265.4 ± 9.5 μM. The manufacturer's assigned values were 250 ± 20 μM for phenylalanine and 277 ± 22 μM for tyrosine.

A series of 50 samples from PKU patients at various levels of effective dietary control, were assayed by the proposed method and by the routinely used fluorimetric techniques (Sigma methods No. 60-F and No. 70-F). A number of these samples were supplemented with added tyrosine, to cover a wider range of concentrations. Correlation between the proposed method and the fluorimetric analyses was excellent over the range of concentrations studied (see Figs. 6 and 7); $r = 0.986$ for tyrosine, $r = 0.976$ for phenylalanine.

DISCUSSION

It was found that optimum separation of tyrosine from phenylalanine was achieved at pH 5.6 but that a stepwise elution system from an initial buffer pH of 3.9 through to a pH of 9.5 was necessary for adequate resolution of these two amino acids from preceding and subsequent plasma amino acid peaks (see Figs. 2 and 4). This ensured return to baseline conditions following elution of phenylalanine and prior to elution of basic amino acids and allowed sufficient resolution of norleucine internal standard from leucine and isoleucine peaks.

Using this system, elution and quantitation of norleucine, tyrosine and phenylalanine is completed within 60 min of sample aspiration. At this time, basic amino acids are still being eluted from the column by the pH 11.50 buffer

so that total removal of all amino acids contained in any sample is complete by 90 min from analysis commencement. In effect, a "slug" of regenerant buffer passes through the resin column between consecutive samples, so that at any given time, amino acids from two samples are being resolved on the same column. The system has been found to be reliable over batches of up to 40 samples with no deterioration in peak separation or resolution and no adverse effects on subsequent full physiological fluid analysis. The method achieves accurate, reproducible analysis of phenylalanine and tyrosine from 100 μl of plasma. The ability of the computer to determine peak area in relation to internal standard up to a sample concentration of 3000 μM, allows accurate determination over a wide range of concentrations, without recourse to further dilutions and repeat analyses.

Internal standard added to the sample at the deproteinisation stage compensates for concentration changes occurring in the protein-free supernatant inherent in this type of sample preparation and also compensates for any small variations in analysis conditions caused by fluctuations in pumping rates, sampling or colour development time. Of previously reported rapid chromatographic analyses of phenylalanine and tyrosine, only the recent system of Ersser [4] achieved sufficient resolution to employ an internal standard, but manual determination of peak height from a recorder trace rather than peak area is used for quantitation.

Although faster analysis systems have been published, such as those of Ersser [4] and Cooke and Raine [3], this system offers improved resolution and the ability to handle a wider range of sample concentrations. In addition, previous systems have been restricted to the analysis of phenylalanine and tyrosine, whereas in the system described here, the same resin column and instrumentation can be used for a full analysis of physiological fluid amino acids, interchange between analysis methods requiring a down time of less than 60 min.

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