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*Short Communication*

# Rapid determination of phenylalanine and tyrosine in urine and serum by HPLC with electrochemical detection

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## Introduction

The hereditary disease phenylketonuria can be diagnosed and its dietary treatment monitored by measuring the phenylalanine concentration in serum or urine. Of the many methods available for this analysis [1], the classical amino acid analyser approach, which involves ion-exchange chromatography and ninhydrin reaction detection, is widely used. Over the years, relatively rapid procedures have been developed (see e.g. refs [2] and [3]); however, the need to use an expensive analyser remains a drawback. In previous work we have shown that amino acids can be detected electrochemically with a copper anode, since complexation of the amino acids with copper ions causes an increase of the anodic current [4]. An amperometric detector for reversed-phase HPLC based on this principle was described [5]. In the present work this detector is applied to the routine measurement of phenylalanine and tyrosine in urine and serum.

## Experimental

The chromatographic equipment consisted of a Waters (Milford, MA, USA) 6000 A pump, a valve with a 20 μl loop and 100 or 120 × 4.6 mm I.D. stainless-steel columns packed with 5 μm LiChrosorb RP-18 (Merck, Darmstadt, FRG). A Waters Model 440 UV detector (254 nm) was used. The amperometric detector was a Metrohm (Herisau, Switzerland) 1096/2 cell with a copper working electrode (diameter 3 mm), operated at +100 mV vs a Ag/AgCl/1 M LiCl methanol–water (1:1) reference electrode, and a platinum auxiliary electrode. A home-made potentiostat/amplifier was used. Experi-

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ments were performed at ambient temperature. The mobile phase was deoxygenated by purging with nitrogen at 30°C. L-Amino acids were obtained from Sigma Chemical Company (St Louis, MO, USA). All other chemicals used were of normal analytical grade quality. Calf serum was obtained from Duphar (Weesp, Netherlands). Urine samples were provided by the Children's Department of the Academic Hospital of the Free University.

## Results and Discussion

*Urine samples*

Using a LiChrosorb RP-18 column and a 0.01 M phosphate buffer (pH = 7.0) containing 5% methanol as the mobile phase, most urinary amino acids elute rapidly, and the non-polar phenylalanine ($k' = 3.35$) is clearly separated from the other amino acids.

Phenylalanine exhibits a UV absorbance maximum at 254 nm, with a molar extinction coefficient of about 200 [6]. UV absorption detection would therefore be adequate for the measurement of the phenylalanine concentrations (10–100 μM) normally present in urine [7]. However, with the simple separation system used, many compounds interfered with the UV detection (Fig. 1), and the phenylalanine signal was hardly visible as a small shoulder on a much larger peak. The superior selectivity of the amperometric detector is obvious. The sensitivity of the detection system for (added) phenylalanine in urine was the same as that in aqueous solutions (Fig. 2a), indicating that poisoning of the electrode by urine components did not occur.

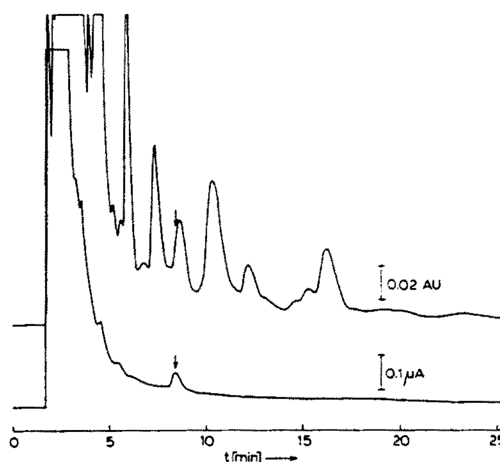


**Figure 1**
HPLC Determination of phenylalanine (arrowed) in a urine sample. Flow rate of the mobile phase, 0.5 ml/min. Upper trace, UV absorbance; lower trace, amperometric detection. Calculated sample concentration: 37 μM.

To study the reproducibility of the method urine samples were injected within an 8 hour period. The relative standard deviations of the peak heights were 6.3% ($n = 9$), 3.4% ($n = 9$) and 2.4% ($n = 7$) for urine samples containing 28, 38 and 53 μM phenylalanine, respectively. Over longer periods, however, the sensitivity varied up to 20%, so daily recalibration was necessary. The influence of the ambient temperature was studied. When the air temperature around the detector cell was raised from 22° to 27°C, the phenylalanine peak heights increased by more than 20%. This strong influence of temperature occurred because the peak height was partly determined by the kinetics of the complexation reaction [4]. Frequent recalibration might thus be avoided by suitable thermostatting of the detector cell.

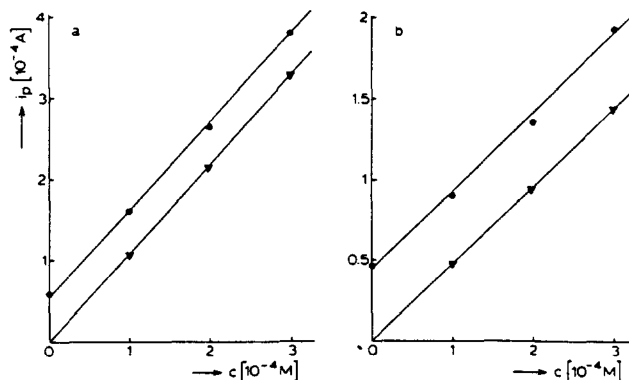


**Figure 2**
Calibration curves for phenylalanine in (a) urine, and (b) deproteinized serum. ▼, aqueous standard solutions; ●, (spiked) samples.

The tyrosine level of urine and serum may also be important in the diagnosis of phenylketonuria [1]. When the methanol concentration of the mobile phase was reduced from 5% to 2.5%, tyrosine was separated from the bulk of the amino acids, though interference of the trailing edge of the major peak still occurred. The reduction of the methanol concentration caused $k'$ for tyrosine to increase from 1.35 to 1.65, and for phenylalanine from 3.35 to 4.10. With standard solutions the detection limit (signal-to-noise ratio 2) of tyrosine was lower than that of phenylalanine (1.0 and 1.5 μM, respectively, under the conditions described). However, due to the greater interferences for tyrosine in urine a poorer detection limit of about 10 μM was observed. Repeated injections of urine samples within an 8 hour period gave relative standard deviations of the peak heights for tyrosine of 5.3% ($n$ = 9) and 3.6% ($n$ = 9), with samples containing 61 and 66 μM tyrosine, respectively.

Urine samples which had been studied on an amino acid analyser were re-analysed for phenylalanine and tyrosine using the amperometric detection system (Fig. 3). With the amino acid analyser peak areas were measured relative to that of an internal standard, whereas with the amperometric detector peak heights were compared with those of a standard solution. For phenylalanine the results obtained with the two methods were in reasonable agreement. The calculated regression line was:

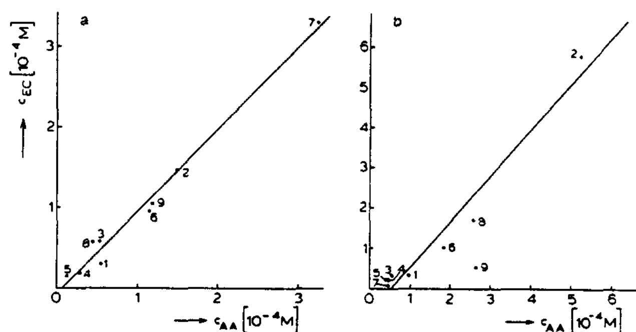


**Figure 3**
Comparison of results obtained with the amperometric detector ($C_{EC}$) and an amino acid analyser ($C_{AA}$) for (a) phenylalanine and (b) tyrosine in nine urine samples.

$$C_{EC} = (1.02 \pm 0.05)\ C_{AA} - (7 \pm 7)\ \mu M,$$

with a correlation coefficient of 0.991. For tyrosine, agreement was less satisfactory, a notable discrepancy between the amino acid analyser and amperometric results occurring for sample No. 9. In this case, however, an unusually broad peak appeared in the amino acid analyser chromatogram, yielding a high result because of an interfering compound. For the other either samples the calculated regression line was: $C_{EC} = (1.14 \pm 0.10)\ C_{AA} - (59 \pm 21)\ \mu M$, with a correlation coefficient of 0.979.

*Serum samples*

To study the effect of deproteination, 1 ml calf serum was spiked with phenylalanine, 0.1 ml of an acetonitrile-concentrated perchloric acid (3:1, v/v) mixture was added, and then 1 ml water. The mixture was shaken for 1 min and centrifuged for 15 min in a bench-top centrifuge. Of the supernatant, 1 ml was neutralized to pH = 4.8 with 0.1 ml 3 M sodium acetate. The total dilution of the serum sample was 1:2.3. The same procedure was carried out for aqueous standard solutions of phenylalanine. The results are shown in Fig. 2b. The recovery of phenylalanine was 99 ± 4% ($n = 9$), at a serum concentration 93 μM.

## Conclusions

Reversed-phase HPLC with amperometric detection using a copper anode is a promising alternative to the use of an amino acid analyser in phenylketonuria screening. The main advantages are the relatively short time of elution (less than 15 min) and the simplicity and low cost of the apparatus used.

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