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Note

Rapid determination of phenylalanine in plasma by capillary high-performance liquid chromatography with electrochemical detection[a]

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Phenylketonuria, a severe hereditary disease due to metabolic disorders and manifested by a high phenylalanine content in the organism, has been diagnosed with the aid of gas chromatography, high-performance liquid chromatography (HPLC) and thin-layer chromatography [1,2]. However, with these methods pre- or post-column derivatization of the analyte amino acids is necessary in order to attain high sensitivity and selectivity of separation. For instance, it has been reported [3] that the determination of phenylalanine in the blood serum of healthy people and patients suffering from phenylketonuria has been achieved by HPLC with fluorimetric detection of dansyl derivatives. A procedure for the determination of phenylalanine in blood serum with the aid of a scanning UV detector has also been reported [4].

HPLC with electrochemical detection has been used for the determination of amino acids, in particular phenylalanine [5–8]. The use of a copper working electrode with a high selectivity for amino acids made it possible to record unsubstituted amino acids by different procedures involving either a conventional column, a microcolumn or capillary HPLC with similar detection limits (*ca.* 1 mg/ml) depending on the nature of the amino acid.

This paper describes the use of a simple chromatographic system combining packed fused-silica capillary columns and a sensitive amperometric detector [9] for the rapid determination of phenylalanine in plasma.

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EXPERIMENTAL

Instrumentation

The chromatographic system consisted of a syringe micropump (Special Design Office of Science and Technology of the Academy of Sciences of the U.S.S.R., Leningrad, U.S.S.R.) and a fused-silica capillary column (50 × 0.34 mm I.D.) packed with a Nucleosil C_{18} sorbent (5 μm) (Macherey, Nagel & Co., Düren, F.R.G.) by a procedure described previously [10]. The end of the column was connected to the coil of an amperometric detector [11]. The working electrode was made of copper. Sample injection was carried out by the flow division method with the aid of a Rheodyne (Berkeley, CA, U.S.A.) Model 7410 valve (volume 0.5 μl) or a valve of volume 120 nl (Special Design Office of Science and Technology of the Academy of Sciences of the U.S.S.R. The chromatograms were recorded with an LKS4-003 recorder (Nauchpribor, Oryol, U.S.S.R.).

Materials and reagents

For eluent preparation, distilled water, ethanol (pure for analysis), potassium dihydrogenphosphate and disodium hydrogenphosphate (chemically pure, Reakhim, Leningrad, U.S.S.R.) and a set of amino acids (Serva, Heidelberg, F.R.G.) were used. The eluent was filtered through a fluoroplastic 0.05-μm membrane filter. Standard amino acid solutions were prepared by the dissolution of individual amino acids in 0.01 *M* hydrochloric acid.

Sample preparation

To 500 μl of plasma were added 500 μl of 10% trichloroacetic acid and the mixture was stirred, left to stand for 15 min in a refrigerator and then centrifuged at 1940 *g* for 5 min. The supernatant was collected and centrifuged again. An aliquot of 500 μl was taken, vacuum dried at 50°C and 500 μl of deionized water were added to the dry residue. The mixture was stirred and vacuum dried until the trichloroacetic acid was completely removed. The dry residue was dissolved in 100 μl of water. A 0.1–0.5-μl aliquot was injected into the column. The sample was eluted with 0.05 *M* buffer solution (pH 7.0) containing 10–30% of ethanol at an elution rate of 3–5 μl/min. The potential of the working electrode was +0.1 V.

Quantification

The calibration against standard phenylalanine solution was compared with the method of standard additions. For this purpose, a 1-ml plasma sample was divided into two halves. One half was prepared by the above procedure. To 500 μl of the other half were added 500 μl of 10% trichloroacetic acid containing 10, 40, 100, 250 and 1000 mg/l phenylalanine, and subsequently the sample was treated as described.

RESULTS AND DISCUSSION

Selectivity of determination

The hydrodynamic voltamperograms of aromatic amino acids (Fig. 1) are of a similar character, and therefore their selective detection is difficult. However, it is easy to separate tyrosine, phenylalanine and tryptophan on a column packed with a reversed-phase sorbent (Fig. 2). Small additions of ethanol to the phosphate buffer solution made it possible to separate completely tyrosine, phenylalanine and tryptophan from other amino acids and to separate phenylalanine from tryptophan and tyrosine.

Fig. 3 shows the selective determination of phenylalanine in blood plasma. Under the above chromatographic conditions, the retention time of phenylalanine is 2 min and the degree of separation attained is sufficient for the identification and determination of phenylalanine. The curve shown by a solid line corresponds to the normal phenylalanine level and the broken line corresponds to the phenylalanine content of patients with phenylketonuria. The total time of a single analysis is 4 min, after which a further sample is injected. To prolong the lifetime of the column, it was washed with methanol after 15–20 analyses.

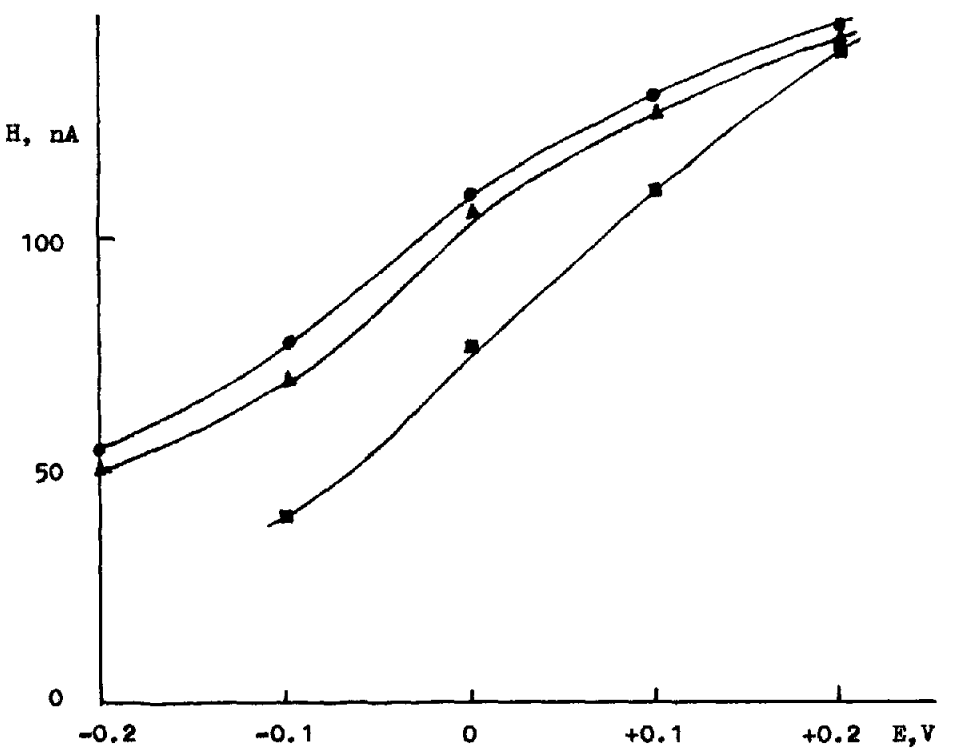

Fig. 1. Voltamperograms of aromatic amino acids on a copper electrode. ● = Phenylalanine; ▲ = tryptophan; ■ = tyrosine.

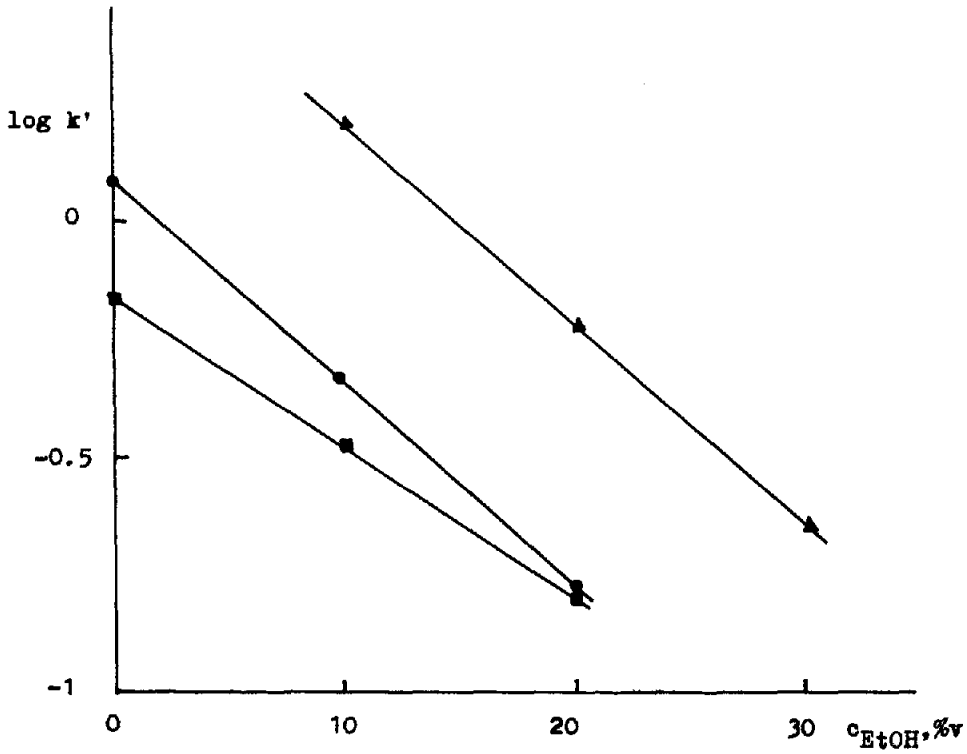

Fig. 2. Capacity factor of amino acids on a Nucleosil C_{18} sorbent *vs.* ethanol (EtOH) content in the mobile phase. Symbols as in Fig. 1.

Calibration

Amino acid concentrations were obtained from the calibration graph (Fig. 4). It is clear that the dependence of the peak height on phenylalanine concentration was linear in the range 0.01–0.25 g/l. In accordance with other workers [3,4], the normal content of phenylalanine in the serum varied from 0.01 to 0.04 g/l, whereas for patients with phenylketonuria it increased to about 0.4 g/l.

The results of the determination of phenylalanine concentrations obtained on the same day by the calibration method with the use of aqueous standard solutions of amino acids and by the addition of phenylalanine solutions of known concentration in 10% trichloroacetic acid to plasma samples coincided, within experimental error. The small difference in the character of the calibration graphs plotted on the first and eleventh days of the experiments was caused by a change in the surface of the copper electrode because it was active and dissolved in the presence of complexing agents [5,8,9].

A considerable decrease in the rate of increase of the signal of the electrochemical detector at relatively high phenylalanine concentrations was due to diffusion

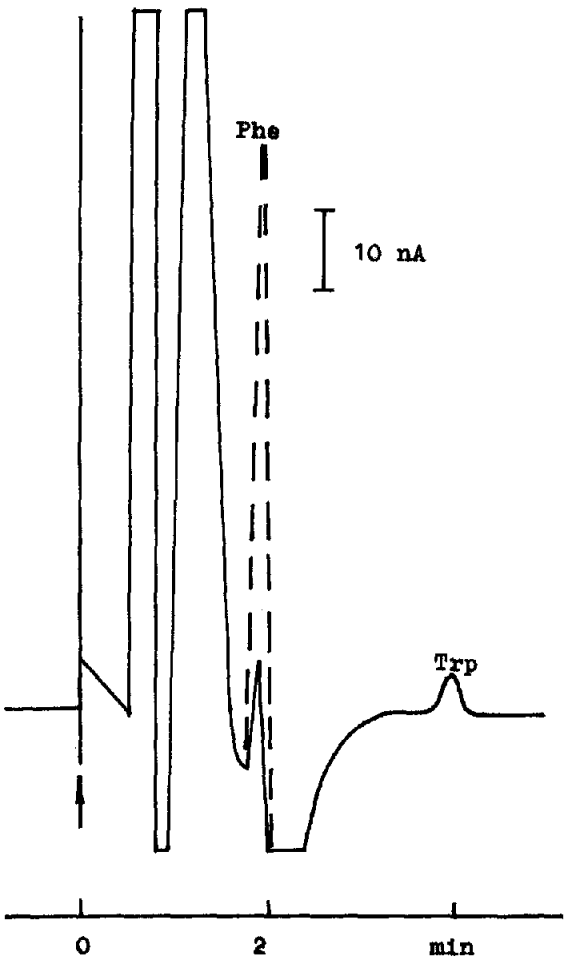

Fig. 3. Chromatogram of a plasma sample obtained by capillary HPLC. Solid line, phenylalanine content under normal conditions (0.03 mg/ml); broken line, phenylalanine content in phenylketonuria (0.32 mg/ml). Column, 50 × 0.34 mm I.D., fused-silica; sorbent, Nucleosil C_{18}, 5 μm; eluent, ethanol–0.05 *M* phosphate buffer (pH 7.0) (5:95); flow-rate, 3.2 μl/min; detector, amperometric detector, $E = +0.1$ V (copper electrode); sample volume, 0.12 μl.

difficulties in dissolution on the surface of the working electrode. This was also confirmed by the existence of a maximum of the dependence of the detector signal on the sample volume (Fig. 5, curve 1), whereas the HETP increases systematically with the volume injected (Fig. 5, curve 2). Here a similar effect of electrode blocking was observed. The peculiar behaviour of the copper working electrode has been reported previously [7,8], a decrease in the current signal of the detector with increasing elution rate, *i.e.*, a decrease in the contact time of the

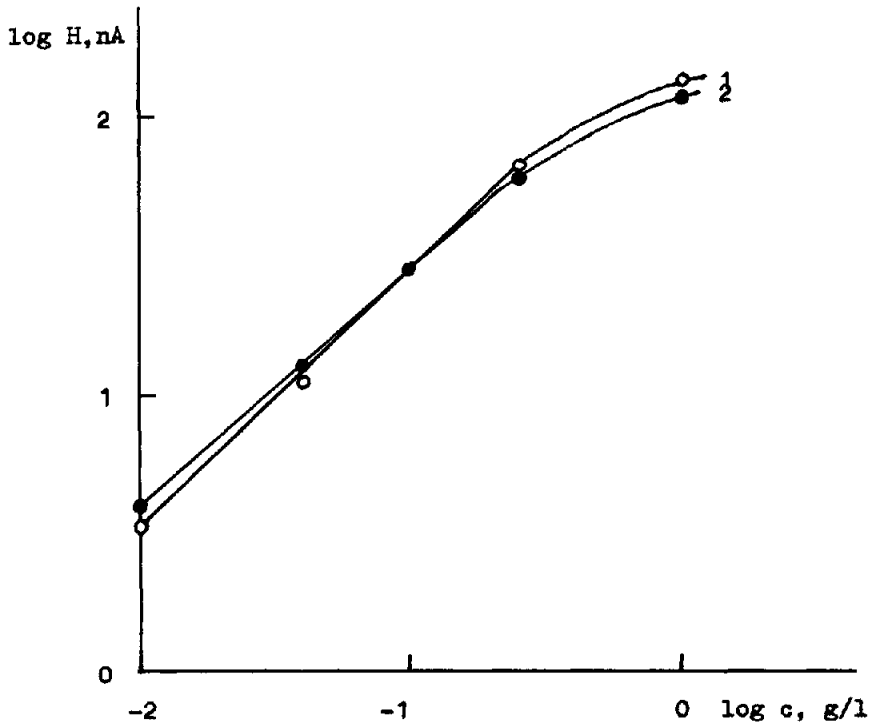

Fig. 4. Calibration dependence for phenylalanine determination: (1) on the first day; (2) after 11 days. Conditions as in Fig. 3.

amino acid with the electrode surface, being experimentally observed. Hence the highest detector sensitivity was attained at a sample volume of 100–200 nl and at a relatively low elution rate.

A study of the dependence of sensitivity on buffer concentration in the range 10^{-4}–10^{-1} *M* did not show any significant effect of concentration on the detector signal.

Precision, reproducibility and sensitivity

The accuracy and reproducibility of phenylalanine determination are given in Table I. The relative standard deviation was 3–7%. The deviations from the theoretical concentration values did not exceed 8%. The reliability of the results for the assessment of phenylketonuria is ensured when the phenylalanine level is one order of magnitude or more higher than the normal value. The detection limit at a signal-to-noise ratio of 2 was 0.01 mg/l.

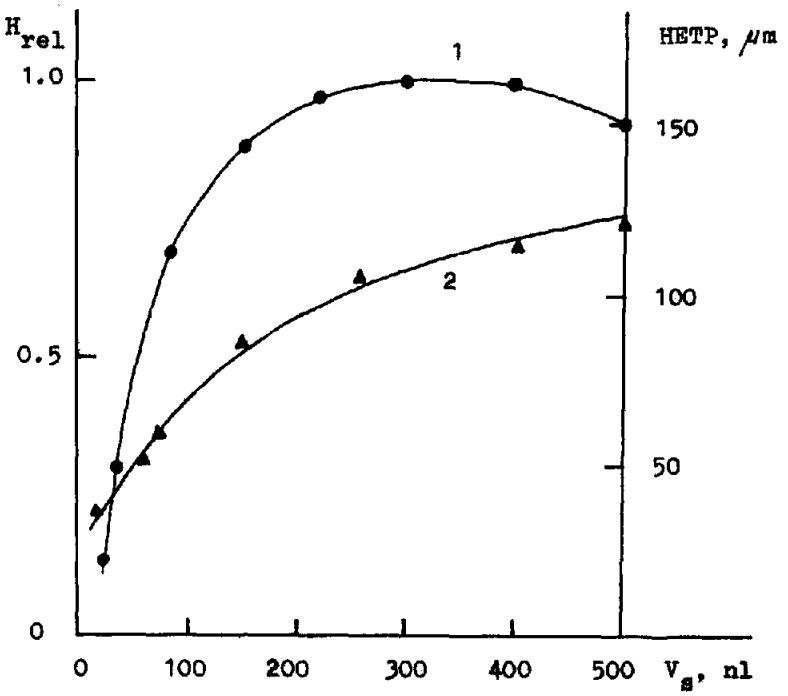

Fig. 5. (1) Relative height of the chromatographic peak and (2) HETP *vs.* sample volume. Phenylalanine concentration 1 g/l; other conditions as in Fig. 3.

CONCLUSIONS

This method for the determination of phenylalanine is relatively simple with respect to instrumentation and economics because it requires only small amounts

TABLE I

ACCURACY AND REPRODUCIBILITY OF THE RESULTS OF PHENYLALANINE DETERMINATION ON THE SAME DAY (n = 3).

Concentration introduced (mg/l)	Concentration found (mean) (mg/l)	Standard deviation (mg/l)	Relative standard deviation (%)	Accuracy[a] (%)
10	9.27	0.6	6.5	92
25	27	0.8	3.0	100
100	104	2.8	2.7	104
500	485	22.6	4.7	97

[a] Accuracy = (found/added) · 100%.

of the sorbent and eluting solutions. The speed of the chromatographic separation and the absence of a derivatization stage make it possible to carry out rapid analyses in pharmaceutical and biomedical research laboratories. The high sensitivity of electrochemical detection with small sample volumes should make it possible to adopt this method chemically, in particular in paediatric clinics, when one blood drop is sufficient for a reliable determination.

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