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Simultaneous high-performance liquid chromatographic determination of amino acids in a dried blood spot as a neonatal screening test[a]

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ABSTRACT

A new screening test on dried blood spots for inherited disorders of amino acid metabolism using reversed-phase high-performance liquid chromatography (RP-HPLC) is described. The method allows the simultaneous analysis of fourteen different amino acids; among these, seven whose blood levels are increased in the most important amino acid disorders have been determined. The procedure requires a preliminary extraction of the amino acids from 9-mm autoclaved dried blood spots by sonication in phosphate-buffered saline. A precolumn *o*-phthaldialdehyde–3-mercaptopropionic acid derivatization is then followed by analysis of the amino acids by RP-HPLC. Blood-spots levels of histidine (His), tyrosine (Tyr), valine (Val), methionine (Met), isoleucine (Ile), phenylalanine (Phe) and leucine (Leu) can be determined in a single 15-min run, including column washing and regeneration. The minimum detectable amount of each amino acid is 0.5 pmol with a linear dose–response range between 1 and 100 μM. The recovery for all amino acids is greater than 70% except for Met (66%). Up to 20 000 samples/year can be processed on a single automated analytical line resulting in an estimated cost of about US$ 0.25/sample. The multiple diagnostic capacity, the low cost and the possibility of complete automation of the method make it suitable for primary perinatal screening of amino acid disorders.

INTRODUCTION

Since 1963, bacterial inhibition assay has been the method of choice for the screening of inherited disorders of amino acid metabolism[1]. The main disadvantage of this test is the inability to detect simultaneously more than one amino acid disorder

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together with the lack of a precise quantification of the levels of the amino acid tested. The possibility of complete automation of the analytical procedure combined with low operating cost represents another necessary characteristic in order to keep the cost/benefit ratio of a screening programme low.

Several methods have been investigated with the purpose of establishing a test suitable for multiple screening of inherited amino acid disorders with high specificity and sensitivity, and the capacity to determine blood amino acid levels accurately; thin-layer[2] and ion-exchange chromatography[3,4] have been the most frequently used methods.

More recently, promising high-performance liquid chromatographic (HPLC) methods for amino acid analysis have been developed. Several techniques, based on precolumn amino acid derivatization in association with reversed-phase (RP) HPLC analysis, have recently been described[5–8].

In this paper we present a micromethod based on precolumn *o*-phthaldialdehyde derivatization of amino acids that allows the RP-HPLC separation of fourteen amino acids; the levels of seven of them (histidine, tyrosine, valine, methionine, isoleucine, phenylalanine and leucine) which are increased in the most frequent aminoacidopathies, were determined after direct extraction from 9-mm dried blood spots.

EXPERIMENTAL

L-Amino acid crystalline salts and *o*-phthaldialdehyde (OPA) were obtained from Sigma (St. Louis, MO, U.S.A.), HPLC-grade solvents from Carlo Erba (Milan, Italy) and 3-mercaptopropionic acid (3-MPA) from Merck (Darmstadt, F.R.G.). The HPLC system consisted of a Pharmacia-LKB (Uppsala, Sweden) 2152 automated gradient controller and two Pharmacia-LKB 2150 HPLC pumps, a Rheodyne (Berkeley, CA, U.S.A.) injector equipped with a 20-μl loop, a Violet (Rome, Italy) T-55 temperature controller, a Kontron (Zurich, Switzerland) SFM 25 spectrofluorimeter, a Kontron 460 autosampler and a Shimadzu (Kyoto, Japan) C-R3A integrator.

Dried blood spots of 9 mm diameter were autoclaved at 120°C for 3.5 min and amino acids eluted by sonication for 1 h in 200 μl of phosphate-buffered saline (PBS) of pH 7.2. The eluate was then subjected to a two-step derivatization procedure: first, 4 μl of OPA–3-MPA [25 mg of OPA in 4.5 ml of methanol plus 0.5 ml of 50 m*M* borate buffer (pH 9.5) plus 50 μl of 3-MPA] were added to 20 μl of the eluate in 100 μl of 50 m*M* borate buffer (pH 9.5); after incubation for 1 min at room temperature, 76 μl of 0.1 m*M* phosphate buffer (pH 6.5) were added followed by the immediate injection of 20 μl of the solution into the HPLC system.

A Spherisorb ODS-2 (3 μm) column (15 cm × 0.46 cm I.D.) was used in conjunction with a Spherisorb ODS-2 (5 μm) guard column (5 cm × 0.46 cm I.D.) (Phase Separations, Waddinxveen, The Netherlands). Both columns were maintained at a constant temperature of 35°C. The flow-rate was 0.8 ml/min and the mobile phase consisted of solvent A, 12 m*M* phosphate buffer (pH 7.2), and solvent B, acetonitrile–water (70:30), mixed according to the scaled composition profile shown in Fig. 1.

RESULTS

Under the experimental conditions used, a good separation of threonine (Thr), histidine (His), citrulline (Cit), alanine (Ala), taurine (Tau), arginine (Arg), tyrosine (Tyr), valine (Val), methionine (Met), isoleucine (Ile), phenylalanine (Phe), ornithine (Orn), leucine (Leu) ad lysine (Lys) was achieved in 13 min (Fig. 1). Among these compounds, our interest is mainly on the determination of the amino acids whose levels are increased in the more frequent aminoacidopathies, *viz.*, His, Tyr, Val, Met, Ile, Phe and Leu.

Different analytical conditions were tested to establish the most efficient extraction of the various amino acids from dried blood spots; the autoclaving time, elution volume and elution time were examined. The minimum autoclaving time required for complete protein fixation to the paper discs was 3.5 min. The extraction of the amino acids from the paper discs was independent of the volume of the eluent in the range 150–400 l, and sonication for longer than 1 h lowered the amino acid recovery, probably because of their degeneration. For a similar reason the eluted amino acids must be kept at 4°C before the derivatization step.

The sensitivity of the method allowed the detection of 0.5 pmol of each amino acid. The dose–response relationship for the seven amino acids in the range 1–100 μM showed a correlation coefficient of greater than 0.999 for all the amino acids tested (Fig. 2).

The recovery of the method was evaluated by adding different amounts of a standard mixture of the various amino acids to a blood sample with known amino

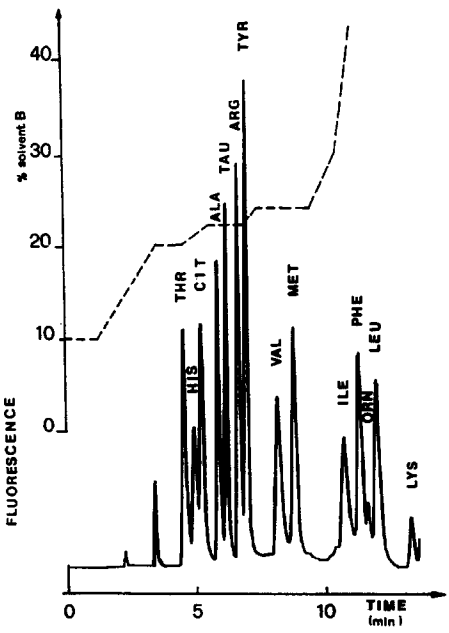

Fig. 1. Chromatogram of a 50 μM standard solution showing the separation of fourteen amino acids. Fluorescence: excitation 270 nm, emission 475 nm.

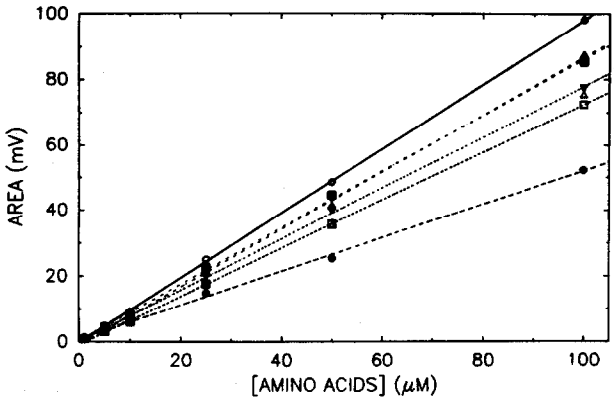

Fig. 2. Linear regression analysis between amino acid concentration and area in mV for (○) Tyr, (●) His, (△) Val, (▲) Met, (□) Ile, (■) Phe and (▽) Leu. The linear correlation coefficients ranged from $r = 0.9990$ for His to 0.9998 for Leu.

acid concentration, followed by spotting on 9-mm paper discs, extraction and analysis as described previously. The mean percentage recovery for each amino acid was calculated at five different concentrations (50, 100, 200, 500 and 1000 μM). The

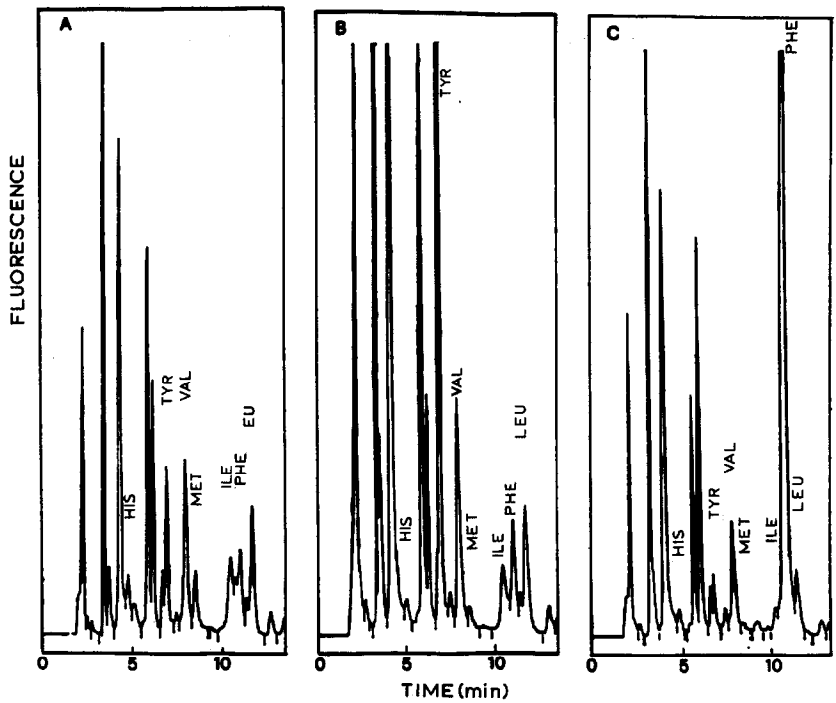

Fig. 3. Chromatograms obtained from (A) a normal newborn and newborns affected by (B) neonatal hypertyrosinaemia (Tyr = 472 μM) and (C) phenylketonuria (Phe = 594 μM). Fluorescence: excitation 270 nm, emission 470 nm.

recovery did not vary significantly in the concentration range investigated; the mean recoveries were His 70 ± 6, Tyr 76 ± 8, Val 87 ± 9, Met 66 ± 6, Ile 97 ± 5, Phe 77 ± 7 and Leu 91 ± 4% (mean ± S.D. for ten determinations at each concentration).

The method was field tested by analysing dried blood spots obtained from a perinatal multiple screening programme for inherited metabolic diseases. As shown in Fig. 3, a good discrimination among controls, phenylketonuric and hypertyrosinaemic newborns was achieved.

DISCUSSION

The basic requirements in a screening programme for inherited diseases are to keep the costs low together with the possibility of screening a wider range of diseases. It is impossible to obtain such a compromise with the traditional bacterial inhibition assay. Inherited disorders of amino acid metabolism have a very different incidence among the neonatal population, from about 1:10 000 for phenylketonuria[9], the most common amino acid disease, to 1:226 000 for maple syrup urine disease[10] and 1:354 000 for homocystinuria[11]. Disorders with such a low incidence are generally not included in screening programmes because of a low cost/benefit ratio.

The micromethod described here allows the simultaneous separation of different amino acids starting from a 9-mm dried blood spot. The method in fact also allows the determination of several other amino acids. It is therefore theoretically possible to screen the neonatal population simultaneously for a wide range of amino acid disorders such as phenylketonuria, tyrosinaemia, maple syrup urine disease, homocystinuria, histidinaemia, urea cycle disorders, hyperornithinaemia and hyperlysinaemia, resulting in an overall probability of detection of an amino acid disorder in at least 17 out of 100 000 newborns screened, without considering neonatal hypertyrosineaemia.

Other HPLC methods applied to amino acid analysis starting from dried blood spots described so far have been oriented towards the identification of tyrosine and phenylalanine[12,13]. These methods are time consuming and cumbersome when applied to large numbers of samples. Most of them require a two-step extraction of the sample by elution of the whole blood from the paper disc followed by amino acid extraction[12–14].

The proposed method allows the direct extraction of amino acids from a large number of dried spots at the same time and the analysis of each sample by HPLC in a total period of 15 min including column washing and regeneration. This accomplishment is important as previously reported methods had analysis times ranging from 8[12] to 20 min[13] for Tyr and Phe or about 80 min for 18 amino acids[14]. The use of an autosampler linked to the analytical line allows more than 90 samples/day to be processed with an estimated cost for each sample of about US$ 0.25.

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