CHROM. 5254

Evaluation of a simple thin-layer chromatographic screening test for amino acid abnormalities

A thin-layer chromatographic test*,[1] was used to screen blood specimens from infants and children for elevations of amino acids to determine its sensitivity for detection of known abnormalities and to note the frequency of positive findings among previously untested patients. The procedure permits chromatographic separation of amino acids in plasma on precoated cellulose plates without deproteinization.

Methods

One-hundred sixty-two specimens were obtained from premature infants in Cincinnati General Hospital at weekly intervals from birth to discharge. Random samples were collected from 196 patients in Cincinnati Children's Hospital. Other subjects included infants suspected of phenylketonuria identified in the Ohio State screening program, patients being treated for phenylketonuria, patients with galactosemia, treated and untreated, and patients previously shown to have histidinemia and hyperglycinemia.

Basic equipment included glass plates, 10 × 20 cm, precoated with cellulose, 2 μl capillaries and holder for application of samples; solvents: *n*-butanol–acetone (1:1) and acetic acid–water (1:2); reagent: 0.4 *M* ninhydrin in butanol–acetone; and a standard solution of amino acids in concentrations corresponding to the upper limit of normal for 5-day-old infants.

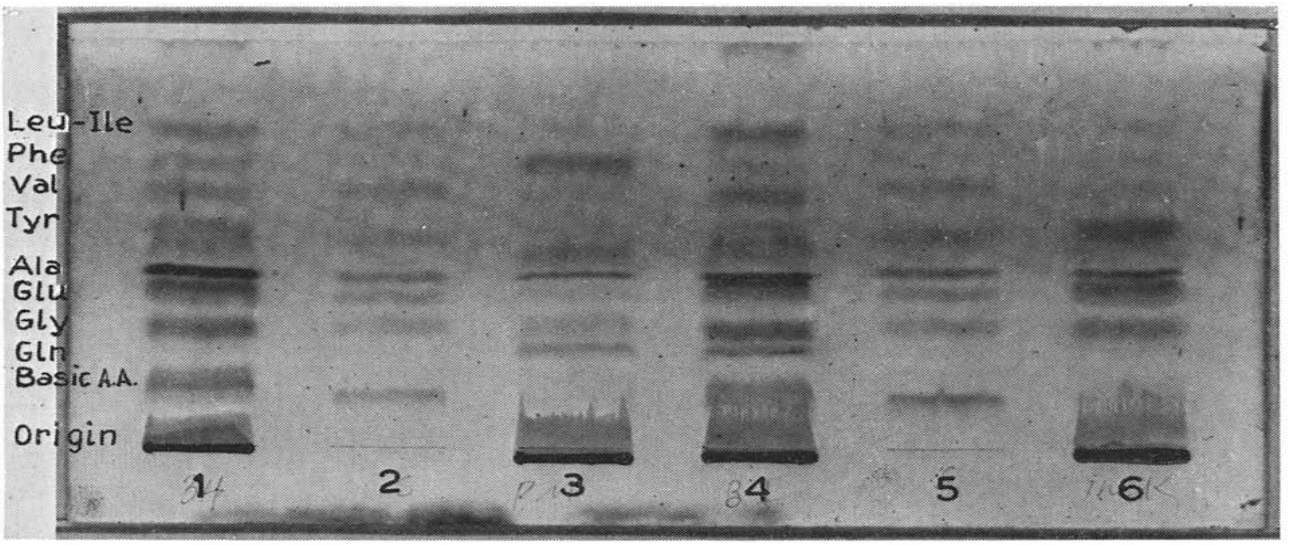

Fig. 1. Thin-layer chromatogram of amino acids in sera and standard. Position 1: specimen with generalized elevation of amino acids including phenylalanine, tyrosine, alanine, glycine. Positions 2 and 5: kit standard (see Table III for composition). Position 3: specimen from untreated phenylketonuric patient with serum phenylalanine (by fluorometry) of 30 mg/100 ml. Note decreased concentrations of other amino acids. Position 4: specimen from infant showing elevations of leucine/isoleucine and valine. Position 6: specimen from infant with untreated galactosemia demonstrating marked tyrosinemia as the only abnormality.

* Diagnostic reagents set for amino acids in plasma (EM-Test TLC), E. Merck, Darmstadt, G.F.R., was obtained through the courtesy of Dr. GUNTER SCHEUERBRANDT.

Blood specimens were collected in microhematocrit tubes. Two microliters of plasma obtained after centrifugation was applied and the plates processed as described below. The plates were marked lightly with pencil for 6 positions 1.5 cm from the bottom edge. The kit standard was applied with the capillary to positions 2 and 5 (see Fig. 1). Specimens of serum were applied to the remaining 4 positions. With practice each specimen could be applied in a uniform streak, approximately 2 cm in length, along the marked position. The plates were placed in a small chromatography tank containing solvent composed of 28 ml of the butanol–acetone mixture and 12 ml of the acetic acid–water solution. Two plates were placed in each tank. When the solvent was within 1 cm of the top of the plate it was removed and dried. Three ml of ninhydrin reagent was added to the solvent in the tank and mixed. The plate was then replaced in the mixture of solvent and reagent for a second run. When the solvent again reached 1 cm from the top the plates were removed, dried in air and heated 3–5 min at 80° for development. Amino acids in the specimens were then compared visually with those in the standard solution. After development with ninhydrin proline was detected by brushing on the plate in a single stroke parallel to the solvent front a reagent containing 0.057 *M* *p*-dimethylaminobenzaldehyde in ethyl acetate–85% phosphoric acid–acetic acid–water (50:6:34:10).

Results

Hyperbilirubinemia did not interfere with separations. Reproducibility of duplicate specimens was good. Hemolysed specimens were poorly separated and generally were not used. An arbitrary scale was set up to permit comparison of the amino acids in specimens with those in the standard which was provided. The scale is illustrated in Table I, showing results of testing specimens with known phenylalanine content. While there was some overlap, the rating scale provided good agreement with measured phenylalanine values. A specimen with phenylalanine rated as 10, is shown in Fig. 1, position 3.

TABLE I

RATING SCALE FOR TLC KIT USING PHENYLALANINE AS EXAMPLE

Rating	*Phenylalanine by fluorimetric determination (mg/100 ml)*
0 = Same as standard	0.5–5
2 = Twice standard	4–9
4 = Four times standard	9–16
10 = Marked elevation	over 20

The rating scale was used to assess specimens from premature infants and from older hospitalized children. Amino acids in specimens from premature infants were generally elevated as shown in Table II, with the alanine, glycine/serine, and basic lines rated at 2 or greater in more than half the specimens. Leucine/isoleucine and valine were elevated in 27% of the specimens from prematures. Tyrosine concentrations at least twice the standard were present in 30% of specimens. In 7% of premature specimens tyrosine was judged to be 4 to 10 times the standard. These speci-

TABLE II

RESULTS OF SCREENING SERUM SPECIMENS FOR AMINO ACID ELEVATIONS

Percent of positive tests: amino acid in specimen at least twice standard.

	Premature infants	*Hospitalized infants and children*
Leucine/isoleucine	27	3
Valine	27	3
Phenylalanine	13	2
Tyrosine	37	1
Alanine	58	5
Glycine	65	5
Basic amino acids	58	4
Number tested	162	196

mens with markedly elevated tyrosine usually had increased levels of phenylalanine as well. Proline test was negative in most specimens. Specimens from a number of premature infants were pooled to obtain a sample for analysis on the amino acid analyzer. Amino acid concentrations in the pooled specimen are compared with the concentrations in the kit standard in Table III. The elevations noted using the arbitrary rating scale were also apparent in the pooled specimen. The aminoacidemia observed in these infants was probably related both to their protein intake (2.5 g/kg/day) and to a relative immaturity of enzymes responsible for metabolism of amino acids. Serial testing of the same infants over a period of several weeks showed that amino acid concentrations gradually decreased to normal.

Table II also shows results of testing random specimens from hospital patients.

TABLE III

COMPARISON OF AMINO ACID COMPOSITION OF POOLED SERUM OF PREMATURE INFANTS WITH KIT STANDARD

	Pooled serum premature (mg/100 ml)	*Composition of kit standard (mg/100 ml)*
Leucine	6.0	3.1
Isoleucine	3.5	1.3
Phenylalanine	4.5	2.5
Valine	5.8	5.0
Methionine	2.7	0.5
Tyrosine	9.0	2.1
Proline	6.1	3.0
Alanine	17.2	4.5
Glutamic acid	9.8	0
Threonine	6.9	5.5
Glycine	7.5	3.0
Serine	7.3	2.0
Glutamine	19.4	0
Arginine	0.6	2.2
Lysine	3.9	4.5
Histidine	2.3	8.5

No instances of specific aminoacidemia were found, that is, no isolated elevation of one amino acid. Glycine was increased more frequently than other amino acids (5% of specimens), but, as in the prematures, glycine, alanine, and the basic amino acids were frequently elevated in the same specimens. Leucine and valine were increased in 3% of specimens, accompanied by glycine, alanine, and lysine so that maple syrup urine disease was not considered. One specimen of 196 tested was considered grossly abnormal with generalized aminoacidemia including methionine which could be seen just below valine, and markedly increased proline.

Among other specimens tested was one from a 1-week-old infant with galactosemia showing an isolated increase of tyrosine. The tyrosinemia disappeared within a few days after removal of lactose and galactose from the diet. An infant born into a family with two phenylketonuric children was tested at three and four days of age and shown to have normal phenylalanine concentration. Another infant with a positive Guthrie test in the newborn nursery showed marked elevation of phenylalanine at two weeks of age (positions 3 and 4, duplicates, Fig. 2). Serum phenylalanine was 40 mg% (fluorimetric determination).

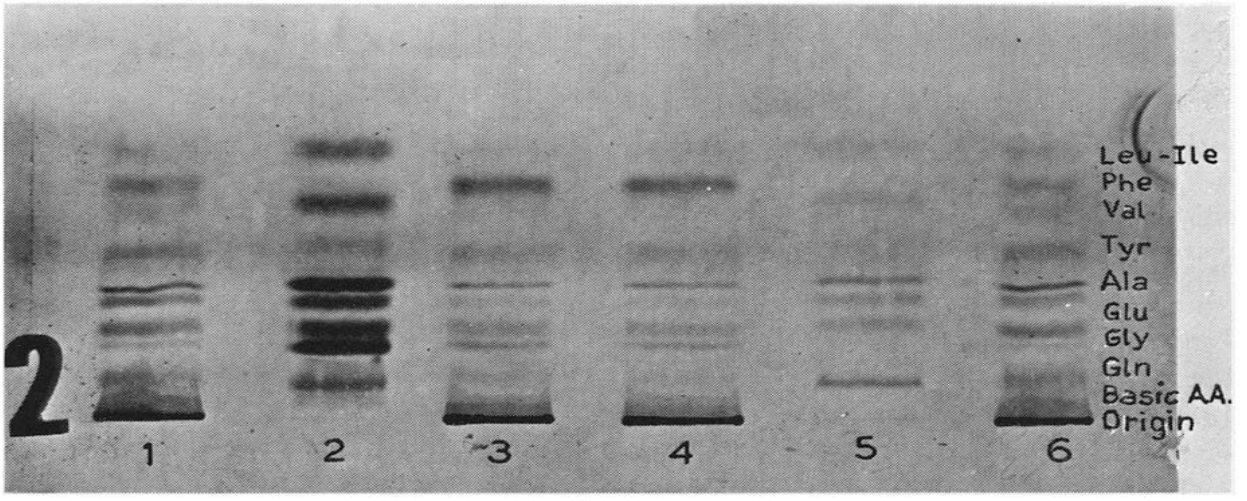

Fig. 2. Thin-layer chromatogram of amino acids in sera and standards. Position 1: specimen from patient with serum phenylalanine of 18 mg/100 ml. Position 2: standard prepared to contain markedly increased concentration (10 times normal) of leucine/isoleucine, valine, alanine, glutamic acid, glycine, and glutamine. Positions 3 and 4: duplicates of specimen from newborn infant with positive test for phenylalanine (by Guthrie method) in the newborn nursery. Serum phenylalanine concentration (fluorimetric method) was 40 mg/100 ml. Position 5: prepared standard from kit. Position 6: mild elevations of phenylalanine and tyrosine in specimens from a premature infant.

Glycine concentrations of 5 to 8 mg% in a patient with hyperglycinemia did not appear elevated compared to the standard or to other specimens. It was not possible to distinguish an elevation in the basic amino acid line in specimens from patients with histidinemia, although histidine levels were 7 to 12 mg%. Glycine and basic amino acids were frequently elevated in otherwise normal specimens, so it was not possible to make the distinction with the test as described. The basic amino acid present was usually lysine, not histidine. The TLC amino acid kit appears to represent a rapid screening procedure for detection of elevations of proline, tyrosine, valine, phenylalanine and leucine in blood serum or plasma. It has an advantage in screening

compared to specific tests for amino acids in that a generalized aminoacidemia can be readily detected.

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A novel charring technique for detection of lipids on thin-layer chromatograms

Lipids are frequently detected on thin layers of silica gel by spraying with a corrosive agent such as sulfuric or chromic acid, followed by charring at elevated temperatures. There are a number of inherent disadvantages to this procedure. The corrosive agents employed pose a health hazard if used in poorly ventilated areas, particularly since they are present in a finely dispersed form. Moreover, it is difficult to spray the plate evenly, especially when viscous reagents such as concentrated sulfuric acid are used. This factor is of prime importance when the plates are subsequently subjected to densitometry and uniform charring of the lipid fractions is desirable. JONES *et al.*[1] overcame the problem of uniform charring of the lipids by exposing the plate to volatile compounds such as sulfur trioxide or sulfuryl chloride; sulfuric acid was then generated *in situ* by exposing the plate to water vapor. However, this procedure is undesirable since volatile, acid-yielding materials are involved. The objections to the use of highly corrosive acids in fine dispersion can be overcome by taking advantage of the thermal instability of ammonium sulfate. At elevated temperatures this compound decomposes yielding ammonia, which is volatile, and sulfuric acid. Sulfuric acid can thus be generated *in situ* by spraying the plate with ammonium sulfate solution (10–20%) and heating. The present communication describes a variation of this procedure in which ammonium sulfate is incorporated into the silica gel layer so that the sulfuric acid generated on heating is evenly dispersed throughout the layer.