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Note

High-performance liquid chromatographic separation of six essential amino acids and its use as an aid in the diagnosis of branched-chain ketoaciduria

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The availability of reversed-phase columns for use in high-performance liquid chromatography (HPLC) has led to the development of newer, more efficient methods for the separation of many classes of compounds. One area which shows great promise is that of amino acid analysis. Classical ion-exchange methods require several buffers, lengthy run times, as well as a separate pump and reaction chamber for post-column formation of either UV-absorbing [1] or fluorescent derivatives [2]. Recently, reports have appeared in which reversed-phase HPLC methods have been coupled with either pre-column [3, 4] or post-column derivatization [5] to provide rapid, sensitive amino acid analysis.

As part of our attempts to develop a rapid reversed-phase HPLC assay for phenylalanine, we have developed a method, modified from Hill et al. [3], to isolate and quantitate six essential amino acids within a 30-min run. This assay effectively separates methionine, valine, tryptophan, phenylalanine, isoleucine and leucine from serum, plasma or other complex physiological matrices and allows quantitation as low as 5—10 pmol injected. Plasma levels performed with this method are in good agreement with standard values performed using ion-exchange methods. The speed, small sample size, and sensitivity of this assay were helpful in the diagnosis and care of a newborn infant found to have an inborn error of amino acid metabolism, branched-chain ketoaciduria.

EXPERIMENTAL

Chemicals

Individual amino acid standards of methionine, valine, tryptophan, phenyl-

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alanine, isoleucine and leucine were obtained from Sigma (St. Louis, MO, U.S.A.) and Schwarz/Mann (Orangeburg, NY, U.S.A.). Physiologic amino acid standards for acidic/neutral and basic amino acids were obtained from Pierce (Rockford, IL, U.S.A.). *o*-Phthalaldehyde (OPA) and β-mercaptoethanol were obtained from Sigma. Acetonitrile, methanol and 2-propanol were of HPLC quality.

Equipment

The HPLC apparatus consisted of two pumps, a WISP multiple sampler, a 10-μm μBondapak C_{18} reversed-phase column, a plotter/printer and system controller all from Waters Assoc. (Milford, MA, U.S.A.). A filter fluorimeter was used (Farrand Optical, NY, U.S.A.) with a 365-nm narrow band pass excitation filter (7-60) and an emission filter transmitting wavelengths > 415 nm (2-A).

Methods

Plasma or serum were diluted with four volumes of 5% trichloroacetic acid (TCA), vortexed, placed on ice for 5 min and then vortexed again. The solution was centrifuged and 0.1 ml of supernatant was taken for derivatization. Individual amino acids were prepared in 5% TCA and physiologic amino acid standards diluted in 5% TCA. The OPA solution (3 mg/ml) was prepared fresh daily in methanol and kept in an amber bottle. β-Mercaptoethanol, 10 μl/ml, was added to the OPA solution. Fluorescent derivatives were made directly in the vials used by our multiple sampler. 100 μl of sample were added to 800 μl of 0.2 *M* sodium borate solution (pH 10.2) and 800 μl of OPA were then added followed by an additional 1.6 ml of methanol [3].

Sodium phosphate, 30 m*M*, was prepared by diluting 60 ml of a stock solution of 0.5 *M* Na_2HPO_4, the pH was adjusted to 7.2 with sodium hydroxide, and diluted to a final volume of 1 l. Solvent A contained the organic solvent, made by mixing 500 ml of 30 m*M* phosphate buffer with 250 ml of acetonitrile and 250 ml of 2-propanol. The solvent was filtered through a Millipore FH filter under vacuum and then degassed. The second solvent (B) was made by diluting the remaining 500 ml of phosphate buffer with an equal volume of glass-distilled water. This solution was then filtered through a 0.45-μm Millipore filter and degassed.

Samples were injected at 30-min intervals and the amino acids were eluted with the following gradient: isocratic at 50% A for 13 min, then increasing the percentage of solvent A by 0.73%/min for 11 min to 58% A and finally decreasing the concentration of solvent A by 2%/min to return to 50% A over the next 4 min.

RESULTS

Fig. 1 is a representative chromatogram showing the elution pattern of methionine, valine, tryptophan, phenylalanine, isoleucine and leucine in a sample of normal plasma. The identity of each amino acid peak was verified by injection of individual amino acid standards. To be certain that no other amino acids co-eluted with these six, derivatives were made of commercial

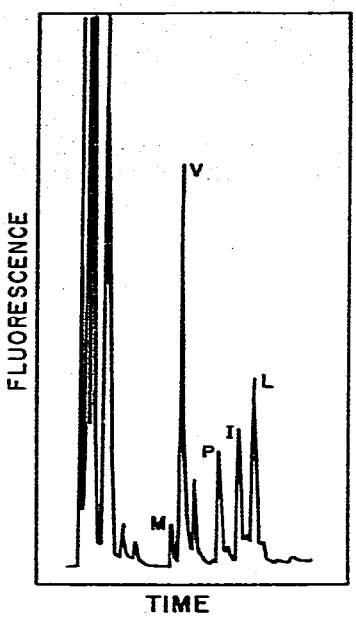

Fig. 1. Chromatogram of normal plasma sample prepared as described in the text. Injection volume, 50 μl. Amino acids detected (pmol injected): methionine (M) (14); valine (V) (81); tryptophan (unlabelled) (20.5); phenylalanine (P) (48); isoleucine (I) (43); leucine (L) (76). To convert pmol injected to nmol/ml plasma, multiply by 1.32.

acidic/neutral and basic amino acid mixtures. In all cases the peaks had response factors equal to those obtained from individual standards.

For each amino acid tested, standards containing 22—735 pmol were injected. For all amino acids peak height was linearly related to concentration ($r > 0.999$) and the intercepts were not significantly different from zero. Standard addition curves were constructed by adding 10, 20 or 40 nmol of individual amino acids to a normal plasma sample which was then derivatized (see Experimental) and chromatographed. In each case the slopes and intercepts of the regression curves were not significantly different from those obtained by the method of external standardization. Recoveries for each amino acid were at least 96% of the actual amount injected.

For the six amino acid standards, the coefficient of variation on multiple injections ranged from 3% (isoleucine) to 6% (tryptophan). The sensitivity of our assay, while not tested directly, may be estimated by examination of Figs. 1 and 2. We feel that quantitation is possible down to levels of at least 5 pmol injected.

The time course of fluorescence decay is noted in Table I. The same sample was automatically reinjected every 3 h over a 21-h period. Peak heights were all normalized, dividing by the peak height at 0 h. The rate of decay is noted in Table I as percentage fluorescence decay per h and ranges from 1.4%/h for isoleucine to 2.8%/h for methionine.

Case report

A 2-week-old female infant was transferred to the Milton S. Hershey Medical

TABLE I

OPA AMINO ACID FLUORESCENCE DECAY

Amino acid standards were reinjected over a period of 21 h. The decay was linear and the slope of peak height/initial peak height graphed vs. time equals fluorescence decay (%/h).

Amino acid	Retention time (min ± S.D.)	Fluorescence decay (%/h)
Methionine	8.46 ± 0.28	2.8
Valine	12.53 ± 0.32	1.5
Tryptophan	14.25 ± 0.45	2.1
Phenylalanine	17.02 ± 0.33	1.6
Isoleucine	25.44 ± 0.23	1.4
Leucine	27.32 ± 0.22	2.4

Center with the diagnosis of bilateral aspiration pneumonia. The infant was the product of a normal uncomplicated pregnancy but the perinatal course was notable for a prolonged labor and fetal bradycardia. At three days of age the infant was breast feeding well and was discharged from the nursery. At approximately one week the mother switched to Similac 20 formula because of the infant's poor suck. During the second week of life the infant continued to lose weight. The mother reported that the child was not active or alert and generally grew more lethargic with time. During the second week

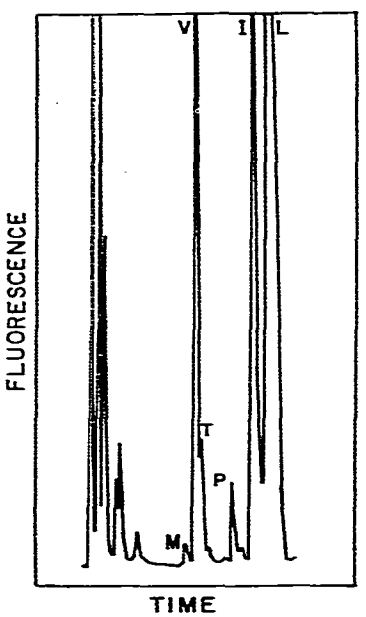

Fig. 2. Chromatogram of a patient with branched-chain ketoaciduria. Sample prepared as described in the text. Injection volume, 50 μl. Amino acids detected (pmol injected): methionine (M) (7); valine (V) (257); tryptophan (T) (7); phenylalanine (P) (33); isoleucine (I) (297); leucine (L) (1859). To convert pmol injected to nmol/ml plasma, multiply by 1.32.

of life her mother began feeding her with a medicine dropper. On the day of admission, while feeding, the child had an apparent aspiration episode and turned blue. She was admitted to a local hospital and then transferred to the Milton S. Hershey Medical Center. Upon admission, abnormal physical findings included a respiration rate in the 40's with mild subcostal retractions and diffuse inspiratory rates. The baby was generally lethargic but responded to painful stimuli. She had a weak cry and a poor Moro reflex. During the hospitalization the infant's neurologic status deteriorated and she developed seizures. Workup was negative for meningitis and evidence of a central nervous system bleed. An electroencephalogram was suggestive of diffuse metabolic encephalopathy. A urine metabolic screen revealed that the urine had a sweetish, maple syrup odor and positive testing for dinitrophenylhydrazine. Blood was drawn and sent for standard amino acid chromatographic analysis, as well as HPLC determination of branched-chain amino acid levels. Within 1 h, the HPLC analysis revealed that the patient's valine, leucine and isoleucine amino acid levels were markedly elevated relative to the control (Fig. 2) and peritoneal dialysis was begun. Dialysis was continued for 96 h during which time plasma samples were obtained by heel stick at 6-h intervals. Table II notes the steady decline of plasma valine, isoleucine and leucine levels during the course of dialysis along with the maintenance of normal phenylalanine levels. Dialysis was continued for 96 h and the infant showed evidence of neurologic improvement beginning 48 h after the start of dialysis.

TABLE II

PLASMA AMINO ACID LEVELS DURING PERITONEAL DIALYSIS

Time after start of dialysis (h)	Amino acid level (nmol/ml)			
	Leucine	Isoleucine	Valine	Phenylalanine
0	2455	392	339	44
6	2600	307	387	29
12	2030	276	352	32
18	1725	245	316	50
24	1441	174	419	61
36	1190	154	340	54
42	1100	166	357	45
48	1020	162	340	45
54	950	167	358	55
60	925	175	375	54
66	880	170	365	50
72	790	140	380	40
78	481	87	229	33
84	635	105	306	53
90	495	69	205	47
96	450	62	200	—
Normal values*	77 ± 21	39 ± 8	161 ± 38	55 ± 10

*See ref. 6, values mean ± S.D.

DISCUSSION

The majority of published HPLC separations utilize non-ionic reversed-phase column packings. Development of amino acid separations have been hampered by the poor selectivity of reversed-phase columns for amino acids. To overcome this problem, separations have been proposed using paired-ion chromatography with post-column detection of amino acids [7] as well as pre-column derivatization with phenylthiohydantoin [8], dansyl chloride [9], fluorescamine [10] and OPA [3]. The OPA derivatives have several advantages including the ease with which they are formed and the extreme sensitivity possible by virtue of fluorescent detection. We initially attempted to perform the separation using the solvents reported by Hill et al. [3]. We substituted β-mercaptoethanol for the ethanethiol used by these authors and as a consequence the elution pattern for phenylalanine and the branched-chain amino acids was altered. In our hands, phenylalanine eluted in the center of a poorly resolved triplet, isoleucine, phenylalanine and leucine. The addition of 2-propanol to the organic solvent has improved our separation considerably, but has increased the operating pressure slightly, from 166 to 186 bar. The time-dependent fluorescence decay of approximately 2%/h for these derivatives requires us to recalibrate with a standard every 3—4 h when processing multiple samples.

The ability of the assay to reproducibly detect picomole amounts of amino acid allows us to utilize small sample volumes for analysis. For example, in the patient presented here, five hematocrit tubes provide sufficient serum for several HPLC runs, a significant advantage where sample size is a problem. This assay is useful for the rapid and sensitive analysis of serum, plasma, urine or other complex biological mixtures. The ease with which samples can be assayed recommends this procedure when amino acid levels must be monitored frequently.

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