with saline or succinate buffer; he also suggested that these proteins then react immediately with BCG as if they were albumin (5).

The method for albumin determination with the Vickers M300 analyzer was modified as follows. Dilute specimens fivefold with distilled water (primary dilutor) and mix a 70-μL aliquot with 1.3 mL of distilled water (secondary dilutor). Incubate the mixture at 37 °C for 9 min, add 1.3 mL of working BCG reagent and measure the absorbance at 640 nm after a further 12 s. The composition of the BCG reagent is as described above.

I compared results by the modified method (12-s reaction) and unmodified method (9-min reaction) with those by the Laurell rocket technique, analyzing 91 plasma specimens from hospital patients. Results are shown in Figures 3 and 4.

In applying the "immediate" reaction to the Vickers M300 multichannel analyzer, the minimum time required for adding BCG to specimens and then measuring the absorbance is 12 s. Gustafsson (5) has reported that acceptable results for plasma albumin by BCG can be obtained if the reaction time is less than 8 to 10 s. Although shortening the reaction time from 9 min to 12 s produces better agreement with the rocket technique, Figure 4 shows that there is still a positive intercept at 10 g/L on the y-axis.

The IFCC Expert Panel on Proteins recommends that albumin should be measured by an immunospecific method, particularly at low concentrations, and that dye-binding methods should only be used as screening procedures (7). Nevertheless, because of their ease of use and application to automated equipment, BCG dye-binding methods are still widely used. In view of this, these methods should be modified to give results that agree as closely as possible with immunospecific methods. I show here that this can be achieved by careful attention to reaction conditions.

References

CLIN. CHEM. 27/1, 146–148 (1980)

Liquid-Chromatographic Quantification of Plasma Phenylalanine, Tyrosine, and Tryptophan

Leonard M. Neckers, Lynn E. Delsi, and Richard Jed Wyatt

Phenylalanine, tyrosine, and tryptophan are isolated and quantified by "high-pressure" liquid chromatography, with fluorescence detection. An isocratic mobile phase and reversed-phase column are used to provide rapid and reproducible measurement of these amino acids in as little as 1 to 2 μL of human plasma.

Additional Keyphrases: heritable disorders screening

Concentrations of the aromatic amino acids tyrosine, tryptophan, and phenylalanine in plasma are of clinical interest for several reasons. Tryptophan participates in the regulation of brain serotonin metabolism (1, 2), and the concentration of this amino acid in plasma has been studied in various disease states (3-6). Data on plasma phenylalanine and tyrosine concentrations have been used in screening for heterozygotes for phenylketonuria (7, 8), and abnormal concentrations have been implicated in several diseases (6).

A technique for monitoring tyrosine, tryptophan, and phenylalanine that is both sensitive and rapid would have clear application in the clinical laboratory. In the past, plasma amino acid concentrations were best measured with an amino acid analyzer, a relatively complicated, expensive, and time-consuming technique (9). More recently, several "high-pressure" liquid-chromatographic methods for several amino acid analyses have been reported (10). We now report a method for the determination of tyrosine, tryptophan, and phenylalanine in the same plasma sample by use of an isocratic elution buffer and fluorescence detection of each amino acid, by virtue of its native fluorescence or by post-column derivatization with o-phthalaldehyde.

Materials and Methods

Chemicals. Unless otherwise stated, chemicals used in preparing the lithium citrate elution buffer and the borate buffer were of analytical grade (Fisher Chemical Co., Silver Spring, MD 20910). o-Phthalaldehyde was from Sigma Chemical Co., St. Louis, MO 63178, and was used without further purification. Perchloric acid (60%, analytical grade) was obtained from Fisher Chemical Co. β-2-Thienyl-DL-alanine was obtained from Sigma Chemical Co.

Apparatus. The chromatograph we used was assembled in our laboratory according to the basic details given by Meek (11). Both the elution buffer and the reagent were contained in individual stainless-steel tanks pressurized by nitrogen gas.
Fifty-microliter samples were introduced into the elution buffer stream by on-line injection, using a six-port high-pressure injector valve (Valco Instruments, Houston, TX 77024). The 25 cm × 0.4 cm column we used was an RP-18 reversed-phase column (Bio-Rad Labs., Rockville Ctr., NY 11571). Fluorescence was detected with a FS 970 fluorometer equipped with a 10-μL flow cell (Schoeffel Instruments, Westwood, NJ 07675). All tubing precolumn was 316-grade stainless steel; all postcolumn (or post-reagent reservoir) tubing was of Teflon.

Sample preparation. Collect venous blood in evacuated blood-collection tubes containing anticoagulant (ACD, NIH Formula "A") and, without delay, centrifuge at 1000 × g for 15 min. Remove the plasma layer and store it at −50 °C until used.

Thaw samples to be assayed and centrifuge them for 2 min at 8000 × g. Mix 100 μL of plasma with 100 μL of 1 mol/L perchloric acid containing 10 mmol of mercaptoethanol per liter and 10 μL of β-2-thienyl-DL-alanine (10 mg in 20 mL of water) as internal standard. After centrifugation, dilute 20 μL of the supernate to 1 mL with water. Inject 50-μL aliquots of this final dilution, equivalent to 0.5 μL of original plasma, into the chromatograph. Inject standards (50 ng per 50-μL injection), prepared similarly, at the beginning and end of each run.

Operation of the Instrument

Phenylalanine. The elution buffer contains, per liter, 0.6 mol of lithium hydroxide and 0.2 mol of sodium citrate and has a pH of 5.0. Pump the buffer at 0.4 mL/min through the heated (55 °C) RP-18 column. Pump o-phthalaldehyde (100 mg in 50 mL of 0.5 mol/L borate buffer, pH 10.3, containing 1 mL of ethanol and 100 μL of mercaptoethanol) at 0.5 mL/min and mix with the column effluent by use of a Teflon T (Altex, Berkeley, CA 94710). Allow the column effluent to mix with o-phthalaldehyde in a 2-m loop of 28-gauge Teflon tubing before entering the fluorometer. The wavelengths used for detection are 330 nm (excitation) and 450 nm (emission), and a 450-nm sharp-cutoff filter is used. One 50-μL sample can be injected every 20 min.

Tyrosine and tryptophan. Conditions identical to those for phenylalanine are used except that o-phthalaldehyde and the mixing loop of tubing are eliminated and the detection wavelengths are 280-nm (excitation) and 330 nm (emission);

---

Fig. 1. Chromatogram of tyrosine and tryptophan (left), and phenylalanine (right) in deproteinized plasma.

Fig. 2. Chromatogram of tyrosine and tryptophan standards (50 and 10 pmol, respectively)

Fig. 3. Chromatogram of phenylalanine standard (50 pmol)
standard solution on the same day, 100 pmol per injection, yielded a CV of 2.1% (n = 10). Repeated assay of the same plasma specimen showed the method to be adequately reproducible (SD 3.6%, n = 8). Analytical recovery, as determined after adding known amounts of amino acid to plasma samples, was 97–100%.

The new method sufficiently resolves phenylalanine, tyrosine, and tryptophan from the other amino acids in plasma. A synthetic standard, containing 21 amino acids (Sigma, kit LAA-21) in equimolar concentrations (100 pmol per injection) was analyzed by our technique. The results were, within experimental error, identical to the quantity of amino acid injected.

Finally, the values we obtain for these three amino acids in normal individuals compare very closely to previously reported values (Table 1).

### Discussion

Of the various methods developed for assay of amino acids in plasma (12, 13), only the amino acid analyzer allows for simultaneous determination of phenylalanine, tyrosine, and tryptophan. With our relatively easy and rapid method, one now can routinely monitor these three amino acids in as little as 1 or 2 µL of plasma in only 40 min.

We have been using this method for 18 months to measure the plasma amino acids in schizophrenic patients; neuroleptic drugs, as well as many others, have no effect on results by the procedure, either in vivo or in vitro, nor have we encountered other problems. The column has only had to be replaced once.

### References