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Rapid and sensitive high-performance liquid chromatographic method for the analysis of tryptophan, tyrosine and phenylalanine in biological samples

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ABSTRACT

In order to overcome problems related to the determination of free tryptophan in biological fluids using conventional methods, we have developed an accurate and reliable procedure based on a specific pretreatment of samples followed by a very rapid and sensitive reversed-phase high-performance liquid chromatographic analysis. The pretreatment consists of adding to the sample of a very low amount of 3 *M* phosphate buffer to maintain pH in the physiological range followed by ultrafiltration. The precision, reproducibility and sensitivity of our method were also evaluated. The recovery of each amino acid was greater than 92%. The use of a microbore column allows the detection of up to 0.2 pmol/μl of amino acid. The method has been applied to the analysis of samples obtained from 25 normal and 10 phenylketonuric subjects.

INTRODUCTION

Tryoptophan is the only amino acid in blood which is bound to a large extent to albumin. Several studies have demonstrated that the degree of binding of Trp to serum albumin is an important factor in the unidirectional uptake of Trp into the brain [1–6]. The binding of Trp to albumin is of non-covalent type and is strongly influenced by blood composition (by its content of fatty acids, drugs and different macronutrients), ionic strength and pH [1,7-10]. For this reason, the usual methods of assessing total Trp content by amino acid analysis do not provide reliable information about the blood level of free Trp [11]. Specific techniques which have been used for this purpose, such as equilibrium dialysis and ultrafiltration, do not meet the requirements of a rapid and sensitive analysis [12,13].

The aim of this study was to establish a rapid and reliable method to determine

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the concentration of plasma free Trp using a new pretreatment procedure followed by a rapid and sensitive high-performance liquid chromatographic (HPLC) analysis with UV detection of tryptophan, tyrosine and phenylalanine.

EXPERIMENTAL AND METHODS

L-Amino acid crystalline salts and other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.) and HPLC-grade solvents from Behring (Riedel-de Haen). The Kontron (Zurich, Switzerland) HPLC system consisted of two HPLC pumps, a 460 autosampler and a 480 column oven, all under computer control; the detection system was a Waters (Millipore, Bedford, MA, U.S.A.) 990 photodiode array detector.

Freshly drawn human blood samples were collected in light-shielded, heparinized vacuum containers to prevent photo-oxidation of Trp and centrifuged at 1085 *g* for 15 min. To prevent pH changes, 10 μl of 3 *M* sodium monohydrogenphosphate (Na_2HPO_4), pH 6.6, were added to 400 μl of plasma to obtain a plasma pH of 7.4 ± 0.01.

A 200-μl sample of plasma was immediately centrifuged at 27 000 *g* for 20 min with Ultrafree M.C. 10 000 NMWL (Millipore), and 20 μl were then injected onto a Spherisorb ODS1 column (5 μm, 25 cm × 4.6 mm I.D.) (Phase Separation, Waddinxveen, The Netherlands). The column was maintained at a constant temperature of 40°C. The elution was performed at a flow-rate of 1.1 ml/min using a gradient mobile phase composed of 12 m*M* phosphate buffer (pH 2.8) and acetonitrile–12 m*M* phosphate buffer (pH 2.8) (50:50, v/v). The eluent composition profile is shown in Fig. 1. For the sensitivity assay we also used a Spherisorb ODS2 microbore column (3 μm, 15 cm × 2 mm I.D.) at a flow-rate of 0.2 μl/min.

The measurement of total Trp was performed by adding 100 μl of plasma to 400 μl of 10% trichloroacetic acid (TCA). The sample was then centrifuged at 14 400 *g* for 15 min. A 100-μl sample of the supernatant was mixed with 100 μl of 0.3 *M* phosphate buffer (pH 7.3) in order to achieve a pH of 2.5 before injecting 20 μl into the chromatographic system.

Tyr, Phe and Trp were quantified using UV absorption at 220 nm.

RESULTS AND DISCUSSION

Changes in the plasma free Trp concentration influence the brain Trp concentration and hence brain turnover of 5-hydroxytryptamine [2,5,9]. Furthermore, a circadian rhythm of Trp plasma values has been reported by several authors [3,9,14]. It is therefore important, in order to perform clinical studies, that there should be available a fast, sensitive and precise method of assaying free and total Trp. The simultaneous determination of Tyr and Phe gives further information on the plasma concentration of neurotransmitter precursors. The method developed in our laboratory allows a good separation of Tyr, Phe and Trp from other UV-absorbing plasma constituents (Figs. 1 and 2).

The dose–response relationship was found to be linear over the ranges 2–100 μ*M* for Tyr and Trp and 10–1000 μ*M* for Phe, and in each case the correlation coefficient was greater than 0.9993.

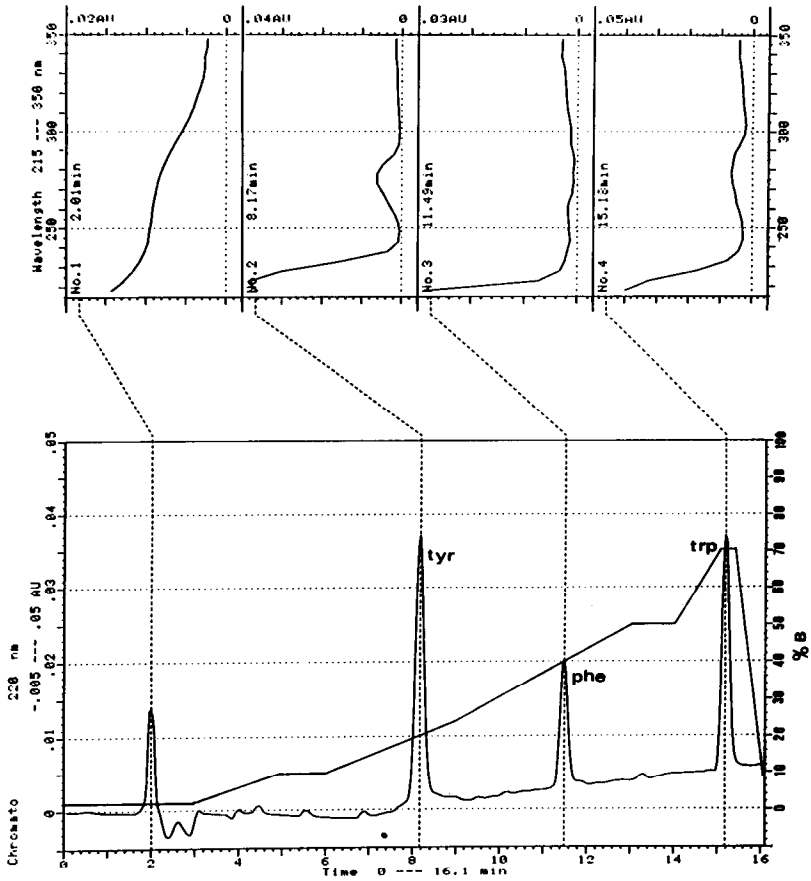

Fig. 1. Chromatogram and spectra of a standard solution containing Tyr (50 μM), Phe (100 μM) and Trp (10 μM).

Using a conventional column, the minimum measurable amount of each amino acid was 2 pmol/μl; by using a microbore column the minimum measurable amount of each amino acid was 0.2 pmol/μl.

In most studies of protein-bound analytes, the stability of pH conditions during sample treatment is critical. This is particularly true in our case; we therefore evaluated the free Trp to total Trp ratio in plasma at different pH and dilutions. Our studies confirmed that there is a significant decrease in free Trp with increase in pH (Table I) and therefore it is necessary to maintain the pH stable within the physiological range during sample treatment. In agreement with Eccleston [12], we also found that increasing the dilution of the plasma with phosphate buffer, under the same conditions of pH and molarity, results in an increase of the free Trp fraction (Table II). These observations indicate that the optimum pH is 7.4 $\pm$ 0.02 at the minimum dilution of the plasma with phosphate buffer (1:1.025).

The method was tested using 25 reference samples taken from healthy consent-

TABLE I

EFFECT OF PLASMA pH CHANGES ON FREE Trp VALUE

Free Trp level variations at different pH were tested by adding 0.3 *M* phosphate buffer, at various pH, to plasma in a 1:1 ratio. Mean values ± S.D. were calculated from five replicated samples.

Sample pH	Free Trp (μM)	Bound Trp (μM)	Free Trp (%)
6.7	31.8 ± 0.2	32.5 ± 0.3	49
7.0	29.5 ± 0.4	34.8 ± 0.2	46
7.3	19.9 ± 0.1	44.4 ± 0.2	31
7.4	17.8 ± 0.3	46.5 ± 0.4	28
7.7	12.6 ± 0.3	51.7 ± 0.6	19
8.5	6.8 ± 0.2	57.5 ± 0.5	10

ing adults after an overnight fast. Plasma total Trp values were 50.4 ± 11 μM; free Trp values were 10.1 ± 5 μM, and the mean free Trp percentage was 20.7 ± 3. Phe mean values were 61 ± 7 μM, and Tyr mean values were 63 ± 8μM. The method was then applied to the analysis of samples obtained from ten hyperphenylalaninemic subjects (Fig. 2).

The values found were in the same range as reported by previous studies [11], except for free Trp. In our case the values obtained were slightly higher than those reported by Eccleston [12] and Flentge *et al.* [13]. Despite the small number of reference samples so far tested, we believe that the small variability observed is satisfactory.

Recovery studies for total Trp were performed by adding different amounts of the amino acid to a blood sample containing a known concentration of Trp. The mean recovery was 97%. This agrees well with other reports. Recoveries of Tyr and Phe were evaluated in a similar manner. The mean recoveries were 102 ± 8% for Tyr and 92 ± 4% for Phe.

Ten samples with known concentrations of Tyr and Phe were analysed by our method and by an ion-exchange amino acid analyser. Good correlations were obtained with both analytes. The correlation coefficient was 0.98 for Tyr and 0.967 for Phe.

TABLE II

EFFECT OF PLASMA DILUTION ON FREE Trp VALUE

Data were obtained by diluting a plasma sample with 0.3 *M* phosphate buffer, pH 7.3. Mean values ± S.D. were calculated from five replicated samples.

% Plasma Dilution (%)	Free Trp (μM)	Bound Trp (μM)	Free Trp (%)
97	10.6 ± 0.3	42.6 ± 1	20
50	13.3 ± 0.2	38.5 ± 0.6	25.7
20	28.1 ± 0.2	23.7 ± 0.4	54.3
11	33.2 ± 0.4	18.6 ± 0.5	64
6	36.6 ± 0.1	15.2 ± 1.1	70.6

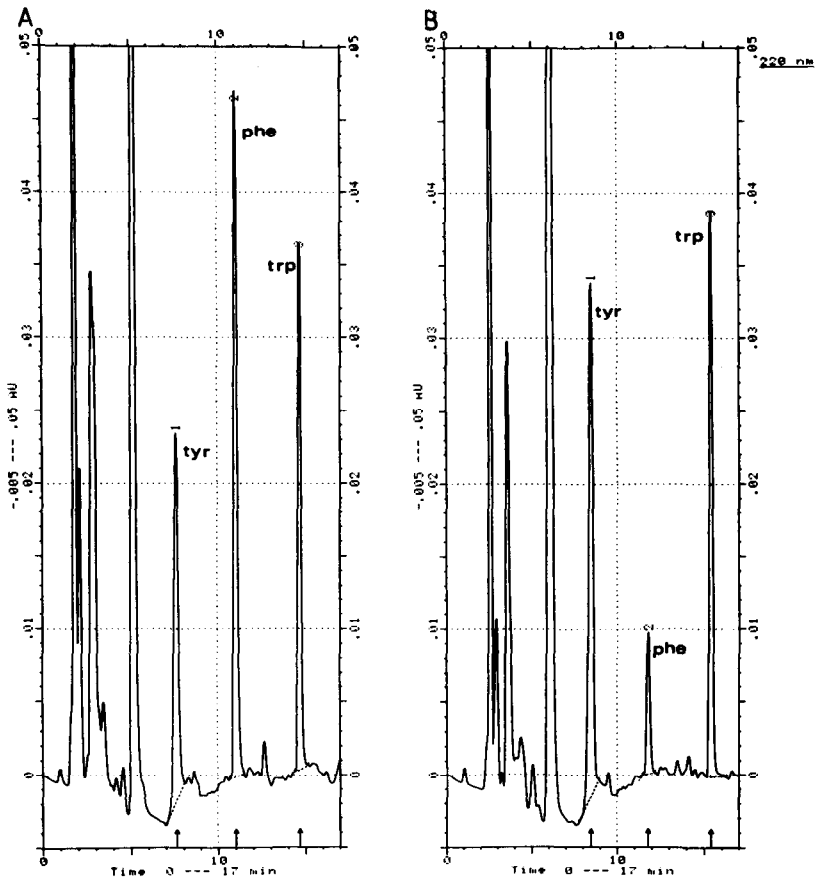

Fig. 2. Chromatograms obtained from plasma analysis of (A) a newborn affected by neonatal hyperphenylalaninaemia (Phe = 304.6 μM) and B a normal subject.

In conclusion, the low level of free Trp in plasma and the particular conditions needed for free Trp determination are strongly affected by Trp photosensitivity [15]. This sensitivity, together with the lability of albumin–tryptophan binding, has prompted us to develop new, more robust methods for Trp determination [12,13]. Most methods take advantage of the high sensitivity of fluorimetric detection, but these methods require more steps for the derivatization of the sample [15–17]. UV detection is far less sensitive but much more practical [18]. We have therefore developed a fast (15 min), precise and sensitive method of determining the concentrations of most important amino acids involved in the synthetic pathway of serotoninergic neurotransmitters.

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