Journal of Chromatography, 380 (1986) 128—132
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3120

Note

High-performance liquid chromatographic determination of total and free tryptophan in serum from control subjects and liver patients

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(First received December 20th, 1985; revised manuscript received February 3rd, 1986)

Previous studies have shown the usefulness of high-performance liquid chromatography (HPLC) for the analysis of tryptophan (Trp) and its metabolites in biological fluids [1, 2].

The procedure described in this paper allows the separation of Trp from interfering endogenous substances in serum or cerebrospinal fluid through a simple deproteinization of samples, but further manipulations are necessary when unbound Trp has to be separated from the fraction bound to albumin. For this purpose, simple ultrafiltration was first proposed [3], but then centrifugation under nitrogen with control of pH was introduced [4] in order to avoid changes in the equilibrium between free and bound Trp.

Given the importance of this consideration, we propose a new HPLC method for detecting and measuring both total Trp and the unbound fraction in serum, previously separated by equilibrium dialysis [5].

EXPERIMENTAL

Equipment

A Varian LC 5000 liquid chromatograph (Varian, Palo Alto, CA, U.S.A.)

0378-4347/86/$03.50

equipped with a UV-50 spectrophotometric detector, operating at a wavelength of 278 nm, and a Varian 9176 recorder were employed. The system also included a Varian CDS 111L integrator.

A 300 × 4 mm I.D. stainless-steel column was slurry-packed in our laboratory with LiChrosorb® RP-18 (Merck, Darmstadt, F.R.G.), following the method of Majors and Hopper [6].

The mobile phase was a mixture of 0.02 *M* sodium phosphate buffer (pH 5.9) and HPLC-grade methanol (95:5) and the flow-rate was 2.0 ml/min.

Reference and internal standards

All chemicals were purchased from Sigma (St. Louis, MO, U.S.A.). Serial dilutions of a standard solution of L-tryptophan in distilled water were prepared in order to obtain concentrations from 0.465 to 15 μg/ml (from 2.25 to 73.45 μ*M*).

To serum samples, 45.1 n*M* (or 22.5 n*M* for free Trp) α-methyltryptophan (internal standard) in 0.1 ml of a 1.0% sodium dodecyl sulphate solution was added, as described in a previous study [1].

Procedure

Samples of blood from healthy volunteers and from patients with overt liver cirrhosis from our institute were examined. To precipitate the proteins, 0.2 ml of 30% trichloroacetic acid were added to 1 ml of plasma containing the internal standard. The mixture was shaken and centrifuged at 2000 *g* for 5 min. The supernatant was filtered through a 0.45-μm HAWP Millipore membrane filter and a 50-μl sample was injected to detect the total amount of Trp [7].

A second fraction of each blood sample was processed in order to obtain the free Trp through equilibrium dialysis, as described previously [5]. A small cellulose dialysis bag (Visking tube 8/32, Serva, Heidelberg, F.R.G.) containing 0.5 ml of an iso-osmotic solution of dextran (6%) was employed. The serum sample (5 ml) was adjusted to pH 7.4 and the test tube, with immersed dialysis bag, was rapidly covered with a layer of mineral oil and incubated for 15 h at 4°C in subdued light. For reduced volumes of serum, the same proportion in the dextran solution was maintained (1:10).

The dialyzed Trp, evaporated under nitrogen and diluted with an appropriate volume of phosphate buffer, was injected in order to determine free Trp.

RESULTS

The reference standard curve as well as the internal standard curve were linear up to 15 μg/ml when both the peak areas and the heights were correlated with known concentrations. The sensitivity was below 0.46 μg/ml (Fig. 1).

The absence of interfering substances was verified by injecting the same sample twice and measuring the absorption at 278 and 242 nm. The absorbance ratio was 2.7 for Trp and corresponded exactly with that of a pure standard solution [7].

Ten replicate analyses of a pooled serum sample from control subjects gave a coefficient of variation of 3.05 ± 1.5% (mean ± S.D.). Chromatograms of total

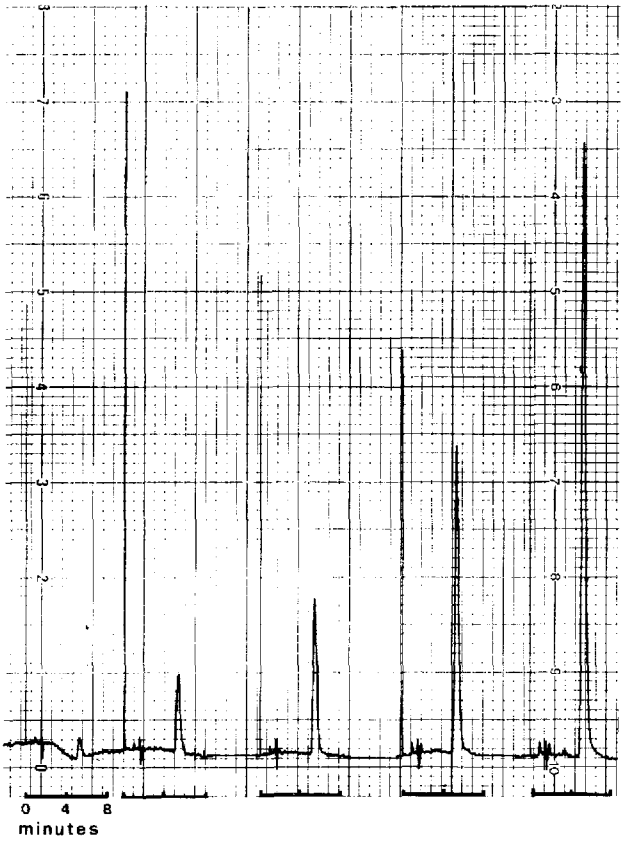

Fig. 1. HPLC profile of L-tryptophan at concentrations ranging from 0.46 μg/ml (2.25 μ*M*) to 15 μg/ml (73.45 μ*M*).

TABLE I

COMPARISON OF SERUM FREE AND TOTAL TRYPTOPHAN IN CONTROLS AND PATIENTS: ANALYSIS OF VARIANCE (F-TEST)

	Tryptophan concentration (mean ± S.D.)				Ratio free/total tryptophan (mean ± S.D.)
	Free		Total		
	μg/ml	μ*M*	μg/ml	μ*M*	
Controls (n = 8)	1.83 ± 0.78	8.96 ± 3.82	8.60 ± 1.69	42.11 ± 8.27	0.22 ± 0.10
Liver cirrhosis (n = 8)	3.81 ± 1.04	18.65 ± 5.09	7.23 ± 1.80	35.40 ± 8.81	0.55 ± 0.18
F-Test (p)	<0.001		Non-significant		<0.001

and free Trp in the serum of a healthy subject and in that of a patient with severe liver cirrhosis are presented in Fig. 2. In the latter case, almost all of the Trp remains unbound to albumin, as observed in the most severe cases of liver disease.

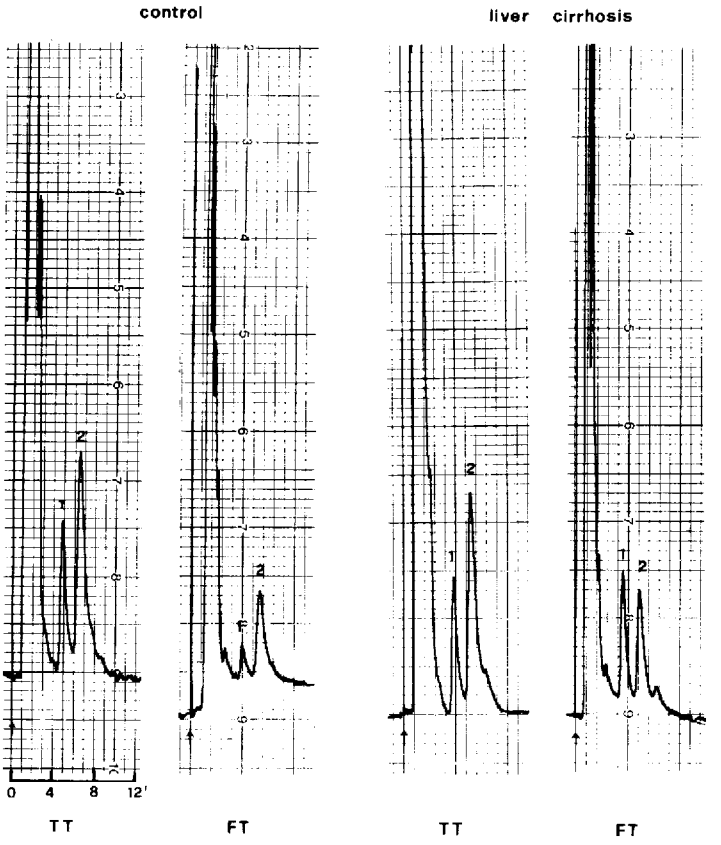

Fig. 2. Chromatograms showing total and free tryptophan in serum samples from a healthy control and from a liver patient. In the latter case, almost the total amount of tryptophan was free. Peaks: 1 = total tryptophan (TT) or free tryptohan (FT); 2 = α-methyltryptophan, the internal standard.

The results of free and total Trp and their ratio in eight healthy controls and eight patients with liver cirrhosis are listed in Table I.

DISCUSSION

The sensitivity and specificity of the proposed high-performance liquid chromatographic (HPLC) method largely satisfy the clinical need for a precise determination of circulating Trp in several pathological and physiological conditions: this is particularly true in liver cirrhosis because of the role of Trp, and mainly of its free fraction, in hepatic encephalopathy induction or worsening [8, 9].

Previous papers proposed different methods for measuring free serum Trp. Among these, the equilibrium dialysis was discarded as time-consuming and cumbersome [10]. Ultrafiltration methods involve centrifugation [11] or air pressure [12], but these procedures can interfere with the equilibrium between the free and bound Trp fractions. For this reason, pH control and displacement of air were proposed [2, 4]. For example, the Bloxam Warren apparatus, coupled

with a fluorimetric detection device, was recently shown to give an incorrect recovery of Trp and a higher coefficient of variation than the HPLC procedures [4].

In particular, the pH control during the entire extraction procedure and the avoidance of air contact seem to be the prerequisites for accurate analysis. For this reason, we chose equilibrium dialysis [5] which, in our experience, gave the best results of free Trp when carried out at 4°C [13].

The mean values of the free Trp obtained with this method agree with those obtained by other groups [4].

The present results confirm a well documented increase in the free-to-total Trp ratio, due to a true increase in the free fraction in chronic liver patients [9], and enable us to pursue further metabolic studies in this field.

ACKNOWLEDGEMENT

This work was supported by Grant No. 84.00884.04 from the "Consiglio Nazionale delle Ricerche", Rome, Italy.

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