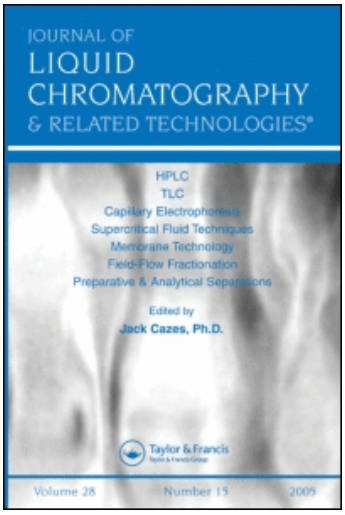

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF TRYPTOPHAN AND SEROTONIN METABOLITES

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ABSTRACT

Serotonin is a neurotransmitter in cerebral centers and its perturbations can produce humor and behavior disorders. In addition, exploration of tryptophan and serotonin metabolism is extremely important for early detection and supervision of treatment in carcinoïd tumors. High performance liquid chromatography (HPLC) enabled us to separate and titrate different metabolites (5-hydroxyindolylacetic acid, serotonin, tryptophan, 5-hydroxytryptophan and N-acetyl tryptophan (NAT) as internal standard) in several types of biological tissues : blood, urine, brain and cerebrospinal fluid. This method is original because it connects HPLC with fluorescence in continuous flow, which allows to change the conditions of pH and buffer. This technique is highly sensitive (below 100 picograms) and very quick (ten minutes).

INTRODUCTION

Serotonin (5-hydroxytryptamine or 5-HT) plays a fundamental part in transmitting nerve impulses within the brain. Changes in metabolism of brain amines involve psychic disorders (6). Since the exploration of brain serotonin in man is difficult, we investigated plasmatic tryptophan (Trp), precursor of serotonin and the factor limiting its synthesis. Trp is absorbed in food, and, in plasma, is linked to albumin (~80 %) ; only the free form is able to pass through the blood-brain barrier (Figure 1).

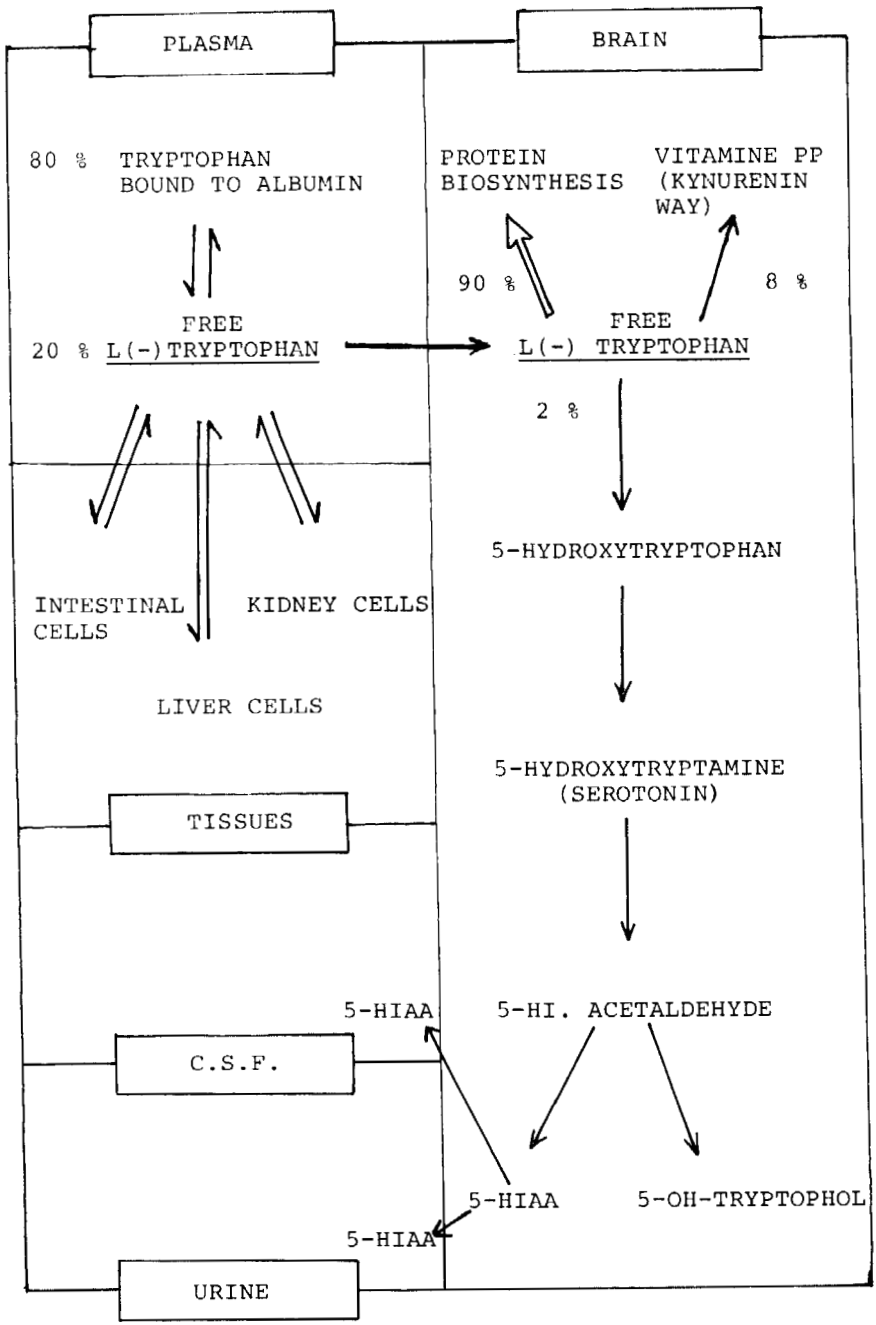

FIGURE 1. Tryptophan metabolism.

Different factors can influence the free Trp level, especially the albumin level (3), the blood pH (3) and certain drugs (1).

Furthermore, measurements of urinary indolylacetic acid (5-HIAA) or, better still, of the blood serotonin, allow the detection of carcinoïd tumors and monitoring of their treatment.

Lastly, there are still gaps in our knowledge of hydroxyindol metabolism at platelet and brain levels. It therefore seemed of interest to possess a sensitive, reliable and rapid technique for measuring Trp, 5-HT, 5-HIAA and other indol derivates like 5-hydroxytryptophan (5-HTP) and 5-hydroxytryptophol in the blood, urine, brain and cerebrospinal fluid of man and animals.

PRINCIPLE

The principle of this method can be resumed in two steps :

1 - Separation of metabolites

This is done by HPLC : the stationary phase consists of a cation exchange silica ; the mobile phase is a solution of $NH_4H_2PO_4$ (pH 2.30).

2 - Spectrofluorimetric detection in continuous flow

On leaving the column, the eluate containing indol derivates is mixed in a continuous flow with a buffer solution allowing to optimize the pH of fluorescence : at pH 6.00, fluorescence ratio is 3 to 6 times (in the function of metabolites) greater than in 2.30 pH medium.

Measuring wavelengths are 301 nm for excitation and 338 nm for fluorescence.

MATERIALS AND METHOD

1 - Materials

a) HPLC

- chromatograph : chromatem 38 (Touzart et Matignon, 8 rue Eugène Hénaff, 94400 Vitry sur Seine, France)

-column : 25 cm x 4.6 mm I.D., Partisil SCX 10 (Whatman, zone industrielle, 45210 Ferrières, France) (10 µm silica beads, with bound sulfonic groups).

b) fluorescence measurement in continuous flow

- peristaltic pump : Technicon (1, route Nationale, 95 Domont)
- autoanalyser II fluorimeter : Technicon
- recorder : Technicon

2 - Reagents

a) HPLC

- mobile phase : $NH_4H_2PO_4$, 10^{-2} M, pH 2.30
- flow rate : 1 ml/min.

b) fluorescence measurement in continuous flow

- Buffer solution (SÖRENSEN) pH 7.00 0.1 M : 8.7 g $Na_2H\ PO_4$, 2 H_2O and 5.6 g. $KH_2\ PO_4$ for 1 L. of distilled water.

3 - Taking of biological fluid and brain samples

a) man

- blood : veinous blood preferably on ACD (citric acid, citrate and dextrose) otherwise on trisodic citrate.
- urine : 24 hours'urine on HCl
- CSF : usual method

b) rats

- brain : dissected in ice after decapitation
- blood : obtained by pressuring the decapited body

4 - Preparation of samples

- CSF and urine were defecated by addition of 1/10 of their volume of 11 N perchloric acid ;
- blood was centrifuged 5 min at 1200 rds/min at 15° C, to obtain a PRP (platelet rich plasma). The PRP is then defecated with perchloric acid under the conditions stated above ;
- rat brain was frozen in liquid nitrogen (30 sec) immediately after dissection, and remained at -15° C until measurement

It was then pulverized in 0.1 N HCl and defecated by 11 N perchloric acid.

5 - Analysis

Samples were centrifuged for 10 min at 3500 rds/min and the supernatant was injected as follows :

- 1 µl for urine of carcinoïd tumor
- 10 µl for others urines
- 50 µl for other samples

Figure 2 shows the analytical system used. Under these conditions, the pressure at the column head was 80 to 90 bars.

RESULTS AND DISCUSSION OF ANALYTICAL CONDITIONS

- This original system makes it possible to optimize separation on the one hand and the detection on the other. Contrary to classical methods, it enables the optimal conditions to be choosen for chromatography as well as for fluorescence (the two sets of conditions being very different), or for other types of detection.

For instance, in this case, the best pH for chromatography was 2.30, whereas the best pH for natural fluorescence was 6 to 10 in function of metabolites.

- Chromatographic conditions : using a solution of $NH_4H_2PO_4$ we made the flow rate vary from 0.1 to 2 ml/min, the molarity from 10^{-1} to 10^{-4} M, and the pH from 1.80 to 6.00.

The following conditions were choosen (Figure 3) :

- molarity : 10^{-2} M
- pH : 2.30
- Flow rate : 1 ml/min

- Spectrofluorimetric detection : optimal conditions were very different. Best pH of fluorescence is 6 for 5-HIAA, 4 for 5-HT, 5 for 5-HTP and 10 for Trp and NAT. Biological levels of Trp are very upper than the others and NAT is the internal standard : so we choose a pH of 6.

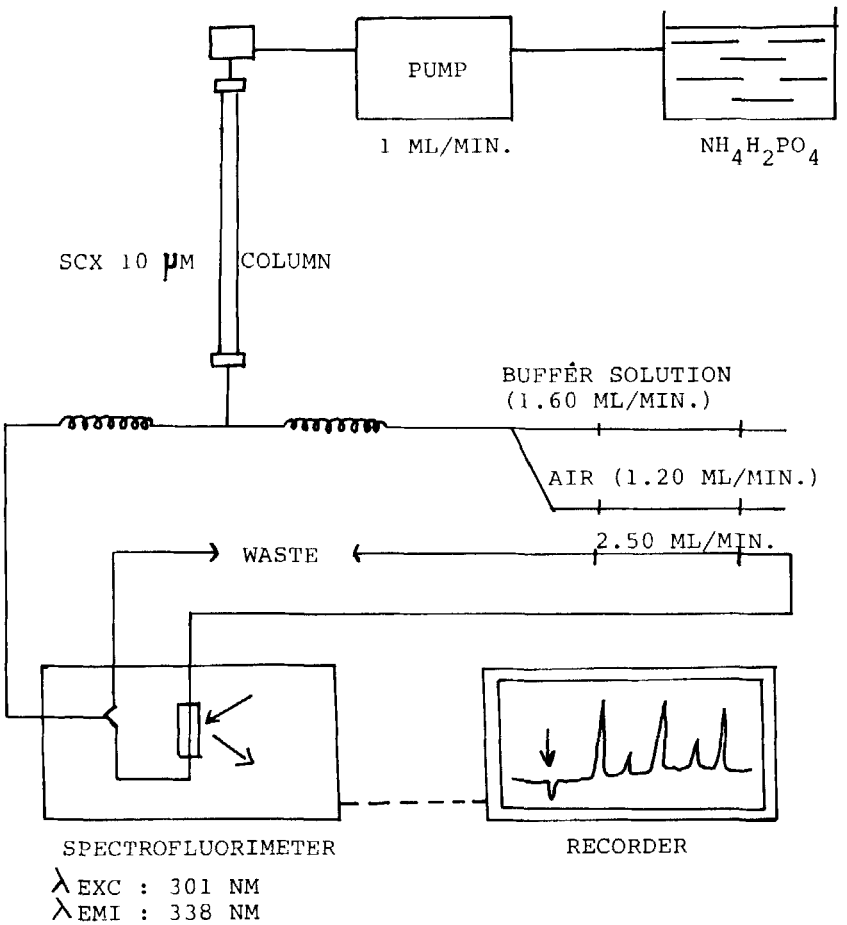

FIGURE 2

HPLC coupled to continuous fluorescence amplification.

- Finally we showed that connecting the two systems in a continuous flow did not affect either efficacity or sensitivity (Figure 4).

- Measurements : we used an internal standard (N-acetyl Trp) for chromatographic injection and double overloading for fluorescence detection.

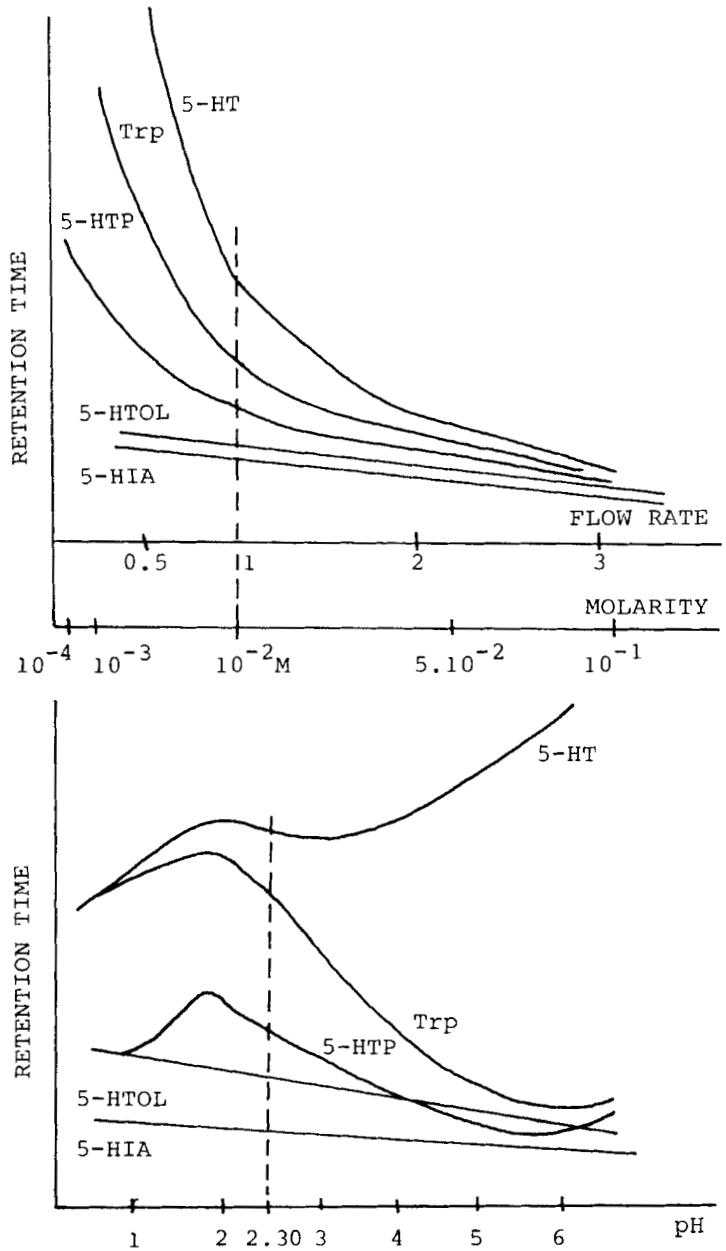

FIGURE 3

Optimization of mobil phase molarity, flow rate and pH.

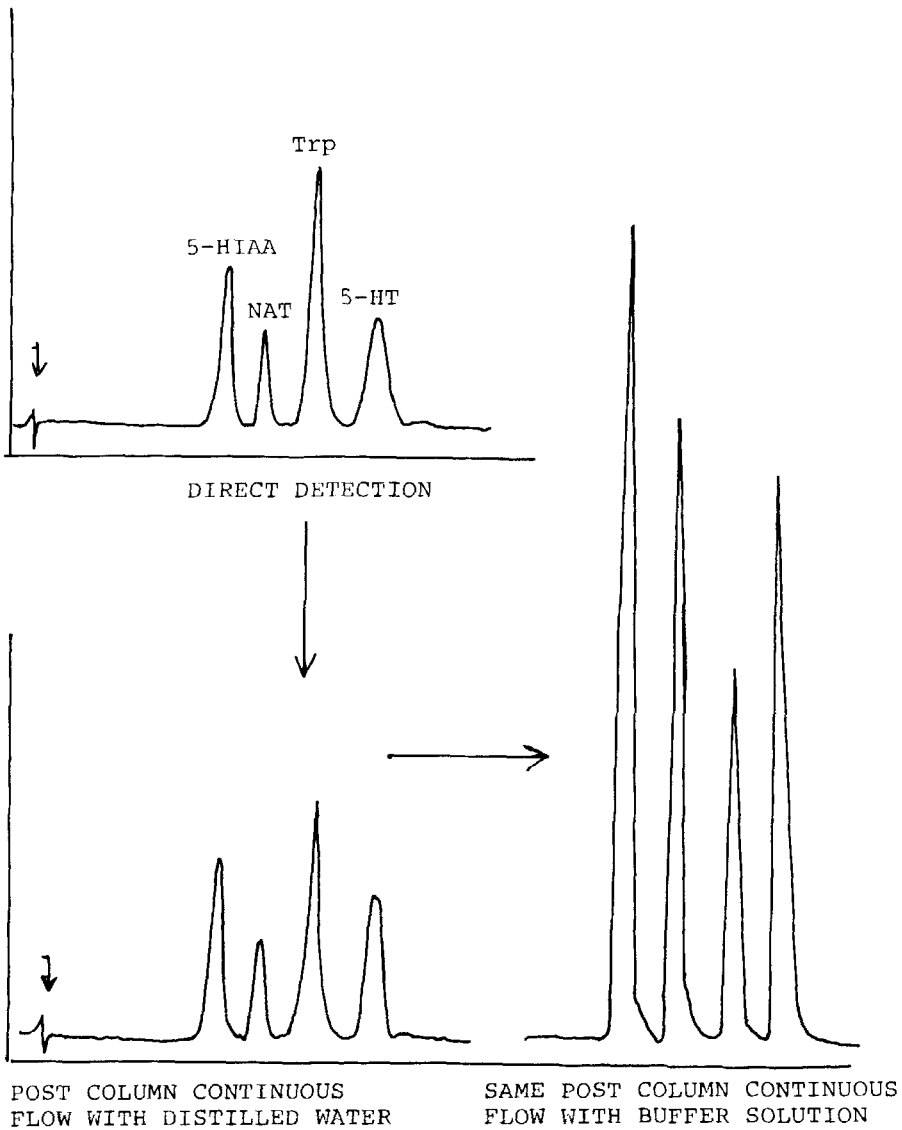

FIGURE 4

Comparison of direct detection with a post-column continuous flow reaction.

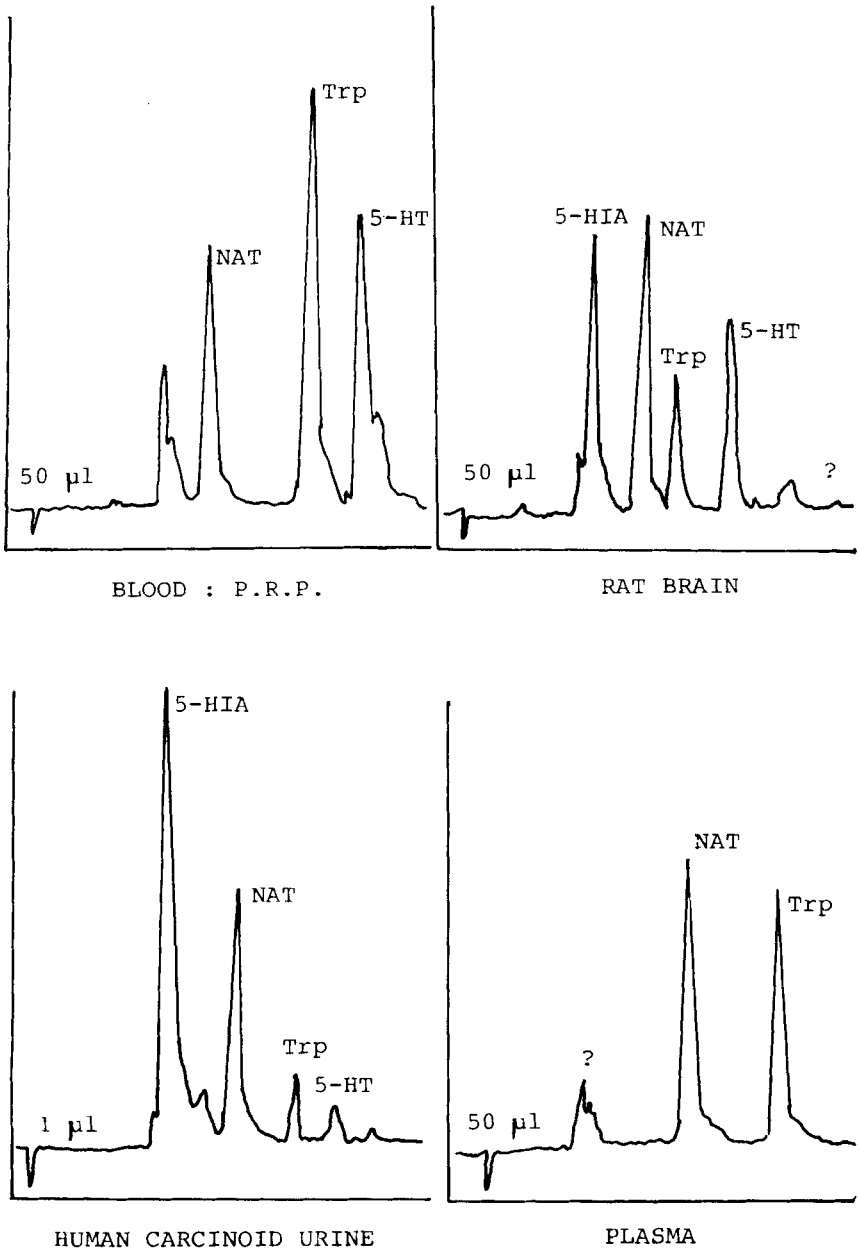

FIGURE 5

Application to biological fluids.

- Linearity was satisfactory up to 50 mg/l, a much higher level than in biological fluids.

- Reproductibility on 20 successive measurements showed a variation coefficient $\leqslant 3$ % in the function of metabolites.

- Accuracy varied from 0.5 to 1 %.

- Sensitivity amplified from 3 to 6 times depending on the molecule enabled us to obtain a detection limit of 100 picograms (5-HT) for 50 µl injected.

- Analysis time remains brief : 10 min for separation and continuous flow fluorescence.

Several examples of chromatograms are given in Figure 5. Reference values for the different metabolites in several biological tissues are currently being established and will be published shortly together with several clinical studies.

CONCLUSION

The essential and original aspect of this method seems to us to be the coupling of chromatography with an independant automatic analytical system. Unlike the conventional techniques this type of detection enabled us to take advantage of the optimal conditions of both the systems involved. It is thus possible to couple with the chromatographic column every kind of analytical reaction, colorimetric, nephelemetric, fluorimetric, etc. This greatly extends the field of HPLC application to molecules which were formerly detected with difficulty.

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