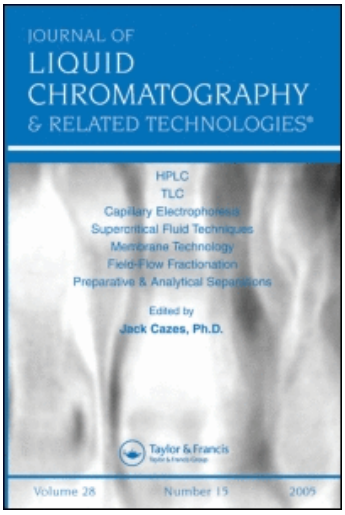

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Simultaneous HPLC Analysis of Tryptophan Hydroxylase Activity and Serotonin Metabolism in Rat Pineal Gland: Determination of Its Kinetic Properties

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SIMULTANEOUS HPLC ANALYSIS OF TRYPTOPHAN HYDROXYLASE ACTIVITY AND SEROTONIN METABOLISM IN RAT PINEAL GLAND: DETERMINATION OF ITS KINETIC PROPERTIES

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ABSTRACT

The determination of tryptophan hydroxylase activity (TPH) in the pineal gland was based upon the separation and detection of 5-hydroxy tryptophan (5OH-TRP) formed from L-Tryptophan (TRP) by high performance-liquid chromatography (HPLC) with fluorimetric detection. Tryptophan hydroxylase activity and TRP metabolites content show circadian variation in the rat pineal gland. The enzyme exhibited a Km value of 53 ± 15 μM and a Vmax of 243 ± 23 pmol 5OH-TRP/min/mg. prot. for L-TRP, and a Km value of 27 ± 4.54 μM and a Vmax of 90.2 ± 4.35 pmol 5OH-TRP/min/ mg. prot. for tetrahydrobiopterine. The present assay is accurate, simple and sensitive, allowing the determination of TPH activity in a variety of enzyme sources. The combination of simultaneous measurements of serotonin, as well as serotonin precursors and metabolites, from a single tissue sample makes it extremely useful for physiological and pharmacological studies.

INTRODUCTION

Tryptophan 5-monooxygenase (hydroxylase) [L-tryptophan, tetrahydropteridine: oxygen oxidoreductase (5-hydroxylating), EC 1.14.16.4], catalyzes the conversion of L-tryptophan (L-TRP) to 5-hydroxytryptophan (5OH-TRP) using tetrahydrobiopterine as a reducing agent and molecular oxygen as a oxidizing agent, and is widely accepted as the rate-limiting step in the biosynthesis of the neurotransmitter serotonin (5HT) (1). A typical circadian rhythm of TPH has been previously described in the rat pineal gland (2, 3), and since it is blocked by propanolol and the enzyme activity increased by isoproterenol and cAMP 4-6), it seems to be under sympathetic regulation as it occurs, *in vitro* and *in vivo,* with serotonin-N-acetyltransferase (NAT) (7, 8). Current methods for measuring TPH activity are based upon the determination of the reaction product, 5OH-TRP, by either fluorimetric (9), radioisotopic (2, 10), or high pressure-liquid chromatographic (HPLC) procedures coupled with fluorimetric or electrochemical detection (11, 12). In the present study we have modified previous separations, in order to be capable of simultaneously measure both TPH activity and tryptophan metabolites within the same pineal throughout a 24 hour cycle. In addition, we have applied the present assay to characterize the kinetic properties of the enzyme in the rat pineal gland.

MATERIAL AND METHODS

Chemicals

L-tryptophan (L-TRP), dithiothreitol, 3 hydroxybencylhydrazine (NSD 1055), catalase, and 5-hydroxytryptophan (5OH-TRP) were obtained from Sigma Chemical Co. (Fancy Road, Poole, England).

Preparation of tissues

Pineal glands were obtained from male Sprague-Dawley rats (300-400 g) maintained in 12:12 h light-dark cycle (lights on, 02:00), with *ad libitum* access to rat chow and tap water. Animals were killed by decapitation at different times and glands were rapidly removed, frozen on liquid nitrogen, and kept at -80°C until assayed. Individual pineals were

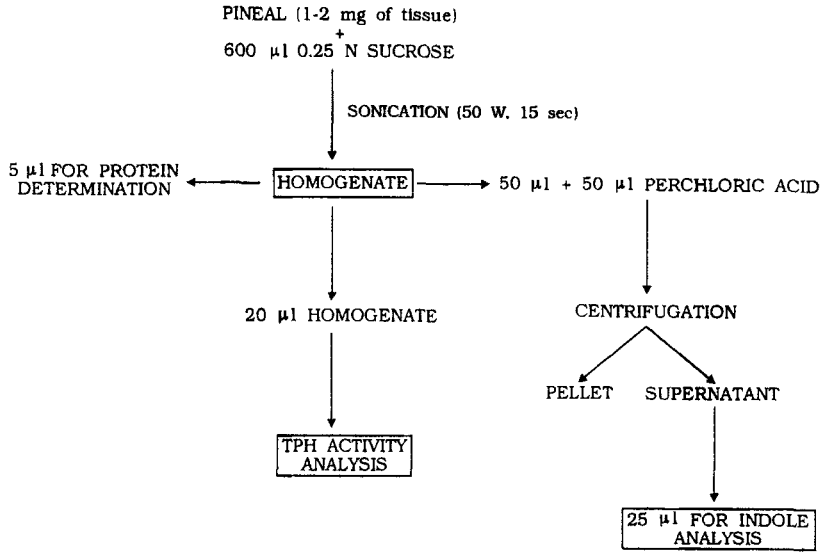

Figure 1.- General sample processing for the analysis, in the same pineal gland, of TPH activity and L-TRP metabolites.

homogenized by sonication (50 W, 15 sec) in 600 µl of 0.25 N sucrose, and different aliquots were used for protein determination, TPH assay, and indoleamine analysis (Fig. 1).

Assay of TPH activity

TPH activity was assayed by measuring the amount of 5OH-TRP formed from L-TRP by high performance-liquid chromatography (HPLC) with fluorimetric detection (FD) (12). Aliquots of 20 µl homogenate were mixed with 18 µl of 1 M tris-acetate buffer (pH = 7.5), 7.5 µl of 6 mM DL-6-methyl-5,6,7,8 tetrahydrobiopterine (THB), 15 µl of 10 mM dithiothreitol, 15 µl of 10 mM NSD 1055, 50 µl of catalase (1462 U), 10 µl of 16 mM ferrous ammonium sulphate, 10 µl of 9 mM L-TRP and 5 µl of destilated water. The general analytical protocol is summarized in figure 2. Briefly, samples were incubated at 37°C for 30 min and the enzymatic reaction was stopped by addition of 20 µl of ice-cold 70% perchloric acid. Following centrifugation at 11.000 rpm for 5 min, 150 µl of 0.05 M carbonate buffer (pH=10.25) and 500 µl of chloroform were added. After vigorous shaking, samples

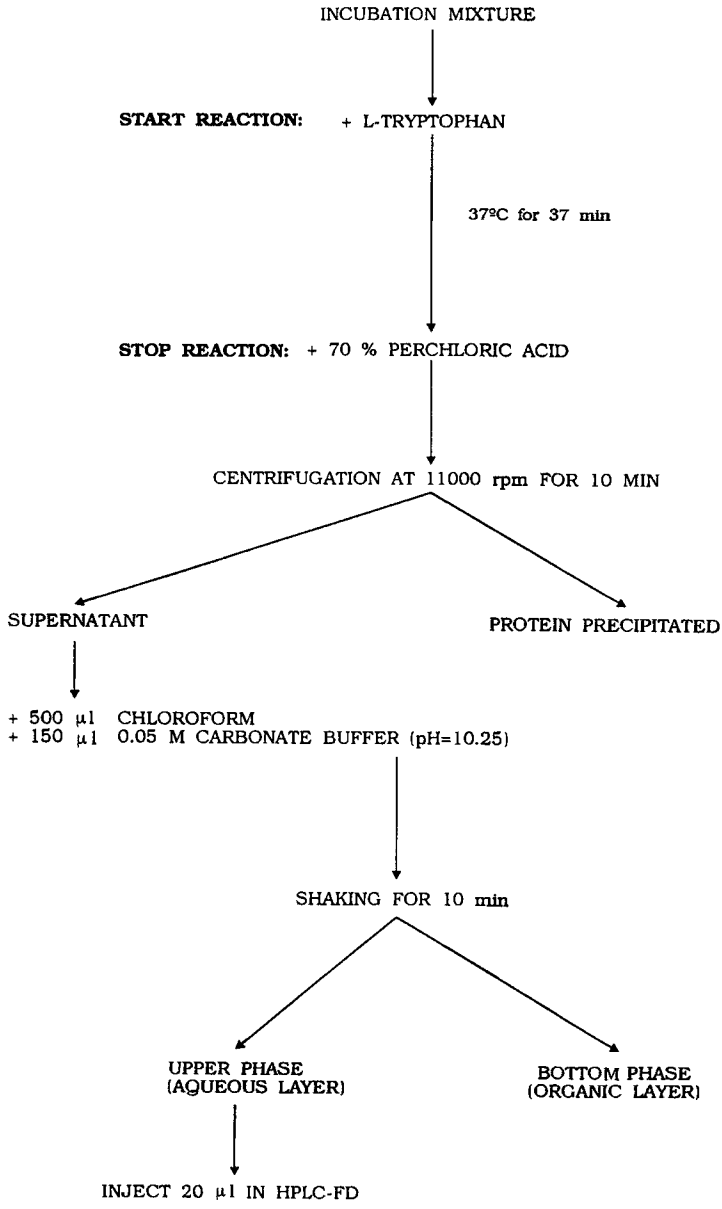

Figure 2.- Diagram summarizing the analytical protocol for TPH activity assay by HPLC-FD.

were centrifuged and 25 µl of aqueous layer (upper phase) were injected into the chromatographic system. The protein content was determined in 10 µl aliquots of the homogenate by the method of Bradford (13), using bovine serum albumin as standard.

In order to carry out the kinetic studies, the final concentration of L-TRP was varied up to 400 µM, while keeping the concentration of THB at a constant level of 400 µM. Conversely, in a different serie of experiments the final concentration of THB varied up to 500 µM, while keeping the concentration of L-TRP at a constant level of 400 µM.

Indoleamine analysis

Fifty µl of the sucrose homogenate were taken into 1.5 ml polypropilene tubes, and 50 µl of 0.4 M perchloric acid/0.025% sodium metabisulfite were added. Samples were then centrifuged at 15,000 rpm for 5 min, and 50 µl aliquots from the clear supernatant were injected into the chromatographic system.

Chromatographic procedure

For both TPH activity and indoleamine content, aliquots prepared as described above were injected into a C_{18} Nucleosil ODS reversed-phase column (particle size 5 µm, Scharlau S.A, Barcelona, Spain). The mobile phase consisted of 0.1 M sodium dihydrogenphosphate, 0.1 mM EDTA, 300 mg/l sodium heptanesulphonate (PIC B7), 5% acetonitrile (vol/vol) in deionized water (Millipore Q System, Millipore Ibérica S.A, Barcelona, Spain), which was adjusted to pH 4.17, and filtered and degassed immediately before used. The system was run at a flow rate of 0.8 ml/min (Waters 510 pump, Millipore Ibérica, Barcelona, Spain), and the fluorescence detector (LS 40, Perkin Elmer Ltd, Buckinghamshire., U.K.) was set at 294 nm and 345 nm as excitation and emission wavelengths, respectively. The identification of peaks by retention time and quantification of 5OH-TRP by peak height was determined using a HP 3396A integrator (Hewlett Packard Co., Avondale, U.S.A.). Under the conditions described above, 5OH-TRP displayed a retention time of 4.2 minutes (Fig. 3), and was completely separated from other potentially interfering substances.

Data analysis

The kinetic analysis of TPH activity was performed by using a nonlinear regression data analysis program (Enzfitter) (14).

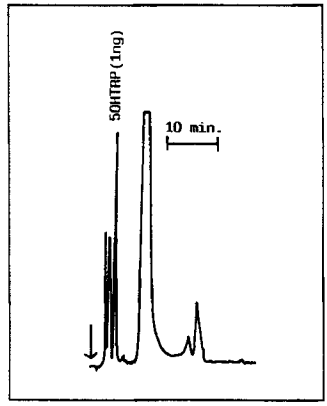

Figure 3.- Representative HPLC-FD elution pattern of 5OH-TRP enzymatically formed from L-TRP in the rat pineal gland. The chromatographic conditions are described in the "Material and Methods" section.

RESULTS AND DISCUSSION

The HPLC-fluorescence method described in this paper provides a rapid, selective, and sensitive approach for the separation and quantitation of the product enzymatically formed from L-TRP, 5OH-TRP, from the rest of compounds present in the enzymatic mixture. The clean up step performed by the addition of organic solvent after stopping the reaction, avoids the presence of interfering substances into analysis matrix, resulting in a complete separation of the reaction product (Fig 3). The correlation between detector response and the amount of authentic 5OH-TRP injected, with and without an organic clean up step, showed a satisfactory coefficient in both cases (0.9886 and 0.9897, respectively) (Fig. 4). The recovery of 5OH-TRP formed enzymatically was 95-97%, and the difference between retention time for 5OH-TRP samples and that of authentic 5OH-TRP was less than 2%. The detection limit for 5OH-TRP, based on a peak height versus background noise ratio of 3:1, was obtained by measuring the native fluorescence and displayed a value of 30 pg/injection.

As shown in figure 5, 5OH-TRP formation increased in a linear manner with increasing amounts of tissue up to 140 μg/tube (Fig. 5A). On the other hand, the rate of

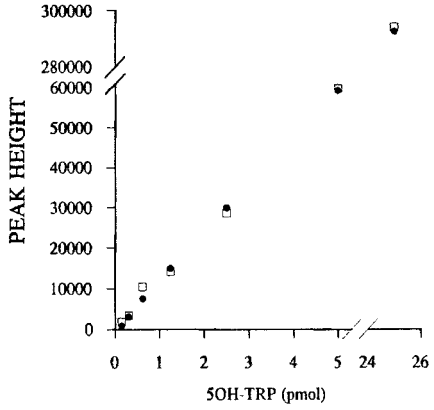

Figure 4.- Linear relationship between detector response and different amounts of authentic 5OH-TRP with (clear squares, r = 0.9886) and without (solid circles, r = 0.9897) an organic extraction with chlorofom.

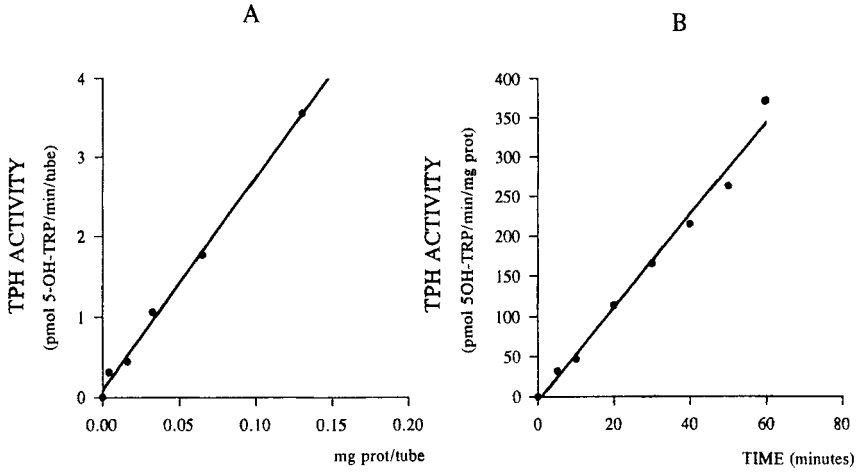

Figure 5.- Linearity of TPH activity with respect to either enzyme concentration or incubation time. Assay was done with incubation time fixed at 30 min, as a function on enzyme concentration (A), or by fixing that variable and then modifying incubation time (B). Standard assay conditions were as described in "Material and Methods". In this and the next figure each point represents the mean of six determinations.

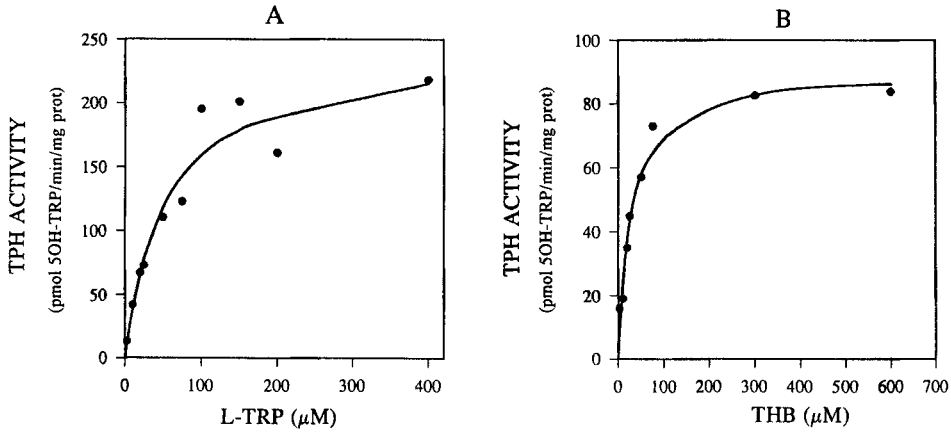

Figure 6.- Michaelis-Menten plots of TPH activity in the rat pineal gland. These studies were carried out while varying the concentration of L-TRP from 2.5 to 400 μM, with a fixed concentration of 300 μM THB (A), or by varying the concentration of THB from 2.5 to 400 μM, with a fixed concentration of 300 μM L-TRP (B).

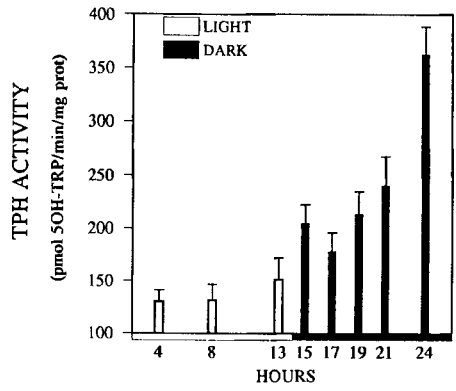

Figure 7.- Circadian rhythm of TPH activity in the rat pineal gland. Groups of animals were sacrifized at the times indicated. Each bar represents the mean ± SEM of eight animals.

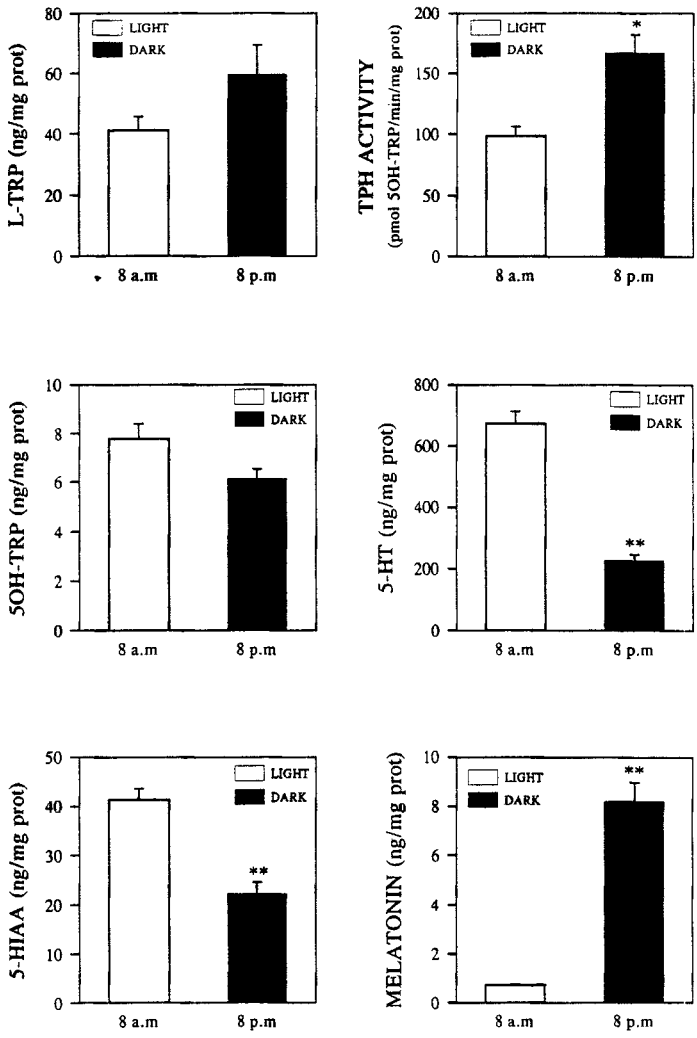

Figure 8.- Pineal levels of L-TRP, TPH activity, 5OH-TRP, 5-HT, 5-HIAA, and melatonin of male rats. The glands were obtained during light (08:00) or dark period (20:00) of a 12:12 hr cycle (lights on, 02:00). Vertical bars represent the mean ± SEM of eight animals.

(*), $p<0.005$ vs Light; (**), $p<0.0001$ vs Light.

product formation was also linear up to 70 min when using a fixed amount of enzyme (Fig. 5B). This allows the use of a wide range of incubation times, as well as the application of the assay to different tissues containing various protein quantities.

The kinetic analysis revealed a Km value of 53.4 ± 15 μM and a Vmax of 245 ± 23 pmol 5OH-TRP/min/mg. prot. for L-TRP (Fig. 6A), a Km value of 27 ± 4.54 μM and a Vmax of 90.2 ± 4.35 pmol 5OH-TRP/min/ mg. prot. for tetrahydrobiopterine (Fig. 6B). These results are consistent with previous data using crude enzyme preparation from rabbit brain (9). We can conclude that the affinity of L-TRP (substrate) and tetrahydrobiopterine (cofactor) for the enzyme is similar. However, the maximum initial velocity, for the same protein concentration, is greater for L-TRP.

Figure 7 shows the variation of TPH activity from pineal glands of male rats collected at different times during a 24 hr period. TPH activity increased gradually from dark onset, displaying highest values at the end of the dark period. This finding confirms a circadian rhythmicity of TPH activity previously reported by others (2, 3, 11), as opposed to early data (16), and supports the view of this enzyme being nocturnally regulated as it occurs with other components of the melatonin synthesizing pathway.

When plotting day and night values of different variables involved in melatonin metabolism, a coherent picture is viewed (Fig. 8). Pineal L-TRP and TPH activity presented a rhythmic pattern similar to that of melatonin, and opposite to that of 5OH-TRP, serotonin (5HT), or 5-hydroxyindole acetic acid (5HIAA). However, as shown in the figure, day/night differences in the content of L-TRP or 5OH-TRP were not significant at the times used in this study, which is in agreement with previous reports in rats (17) and rabbits (18).

In summary, the present assay offers a simple and reliable method for measuring TPH activity in homogenates from rat pineal glands and other tissues. The assay is accurate and highly sensitive, allowing the determination of TPH activity as well as several TRP metabolites within individual pineals with the same chromatographic system. This approach also allows us to reduce the number of animals needed for physiological or pharmacological studies, and provides a way to correlate circadian or experimentally induced changes in various components of the serotonin metabolic pathway. In addition, this method has been proved useful to detect low levels of TPH activity, not only in homogenates from rat pineal glands, but also in other enzymatic sources, such as rat dorsal raphe and retina (data not shown), which contain small amounts of neural tissue.

ACKNOWLEDGMENTS

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