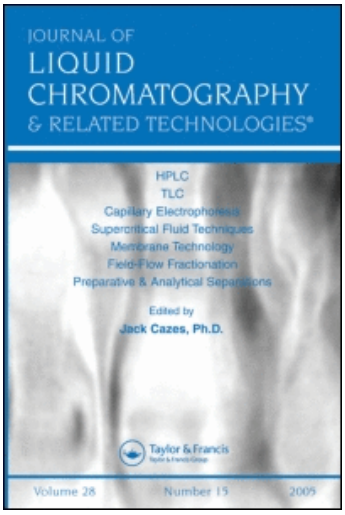

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FLUOROMETRIC DETERMINATION OF TRYPTOPHAN AND ITS BRAIN INDOLEAMINE METABOLITES BY ION-PAIR HPLC

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ABSTRACT

The reverse phase HPLC separation of tryptophan, serotonin, 5-hydroxyindoleacetic acid, tryptamine and indoleacetic acid using two solvent systems (one of them containing an ion-pairing reagent) is reported. The two low concentration eluents employed are either formic acid/water or 0.005 mol/L solution of 1-pentane sulfonic acid. In both cases chromatographic separation was achieved through a linear gradient elution using methanol/water (7/3) as the high concentration eluent. We also describe the variation of retention volumes of these compounds as a function of the pH of the mobile phase. pH values ranged from 1.5 to 4.0 and were obtained by adding either formic acid or NaOH respectively to the low concentration eluent. Tryptophan and its metabolites were detected fluorometrically. All compounds show a linear response in the pg to μg range. The chromatographic separation achieved allows a concurrent measurement of tryptophan and its main indoleamine metabolites which coupled to the high sensitivity of fluorometric detection permits a direct estimation of these metabolic pathways in brain tissue.

INTRODUCTION

The study of tryptophan (TP) and its neuroactive indole metabolites (Fig. 1) has attracted a growing interest in recent years due to the involvement of

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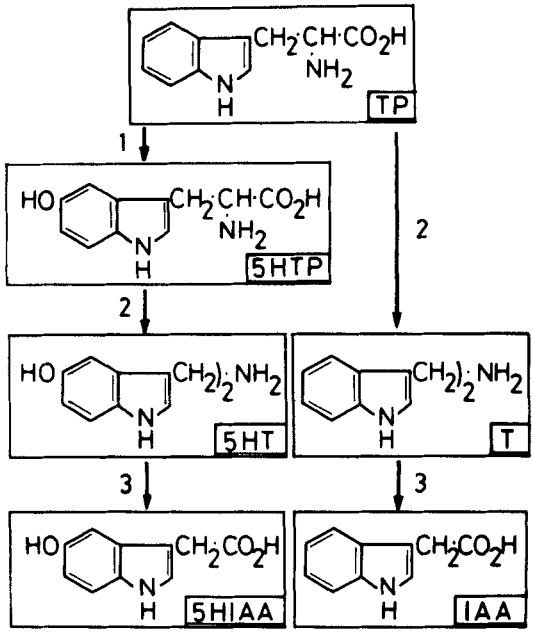

FIGURE 1. Metabolic indole pathway of tryptophan. The catalyzing enzymes are numbered as follows: 1. Tryptophan hydroxylase, 2. Aminoacid decarboxylase, 3. Monoamino oxidase.

these compounds in a wide variety of physiological and pathological states (altered levels have been described in many diseases as well in severe mental disorders (1)). Accordingly, the quantitative analysis of TP metabolites could be a helpful tool to clarify the etiology and treatment of these diseases.

Serotonin (5-HT) is at present one of the few well-established central neurotransmitters. Conversely, tryptamine (T) has not been unequivocally detected in brain tissue until recently (2), and its exceedingly low con-

centration (less than 0.5 ng/g in rat brain tissue) seems to argue against a classical neurotransmitter role in the mammalian central nervous system (CNS). However, it shows several interesting pharmacological properties (3,4), recent data (5) suggesting that T could act in the CNS as a modulator in serotonergic neurotransmission.

The catabolism of both indoleamines 5-HT and T leads to 5-hydroxyindoleacetic acid (5-HIAA) and indoleacetic acid (IAA) respectively. Their concurrent measurement allows an estimation of the turnover of these substances and the interrelationships of both pathways. To date most analytical approaches have been almost exclusively dedicated to 5-hydroxyindoles, 5-HT and 5-HIAA, (6) ignoring the non-hydroxylated analogues T and IAA. However, TP indole metabolites can be determined by several techniques, such as the radioisotopic (7) and mass fragmentographic (8) assays. Recently the need to simplify analytical procedures has estimulated the development and improvement of HPLC methods with electrochemical (6,9), ultraviolet absorbance (10), and fluorescence (11) detectors. In spite of this, the power of the modern HPLC-fluorometric systems has not been exploited to its full extent, especially since most papers dealing with the application of HPLC to the assay of biogenic amines do not consider -with few exceptions (12,13)- the existing possibilities for the concurrent determination of all the most significative components of the brain indoleamine pathway. For such a purpose, one would need a chromatographic system capable of resolving compounds of a widely different nature, such as in this case the aminoacid precursor and its related amine and acid metabolites, as well as a detection method capable of providing sufficient specificity to detect only those substances of interest.

Along these lines and taking full advantage of the characteristics of modern liquid chromatography for the separation of polar compounds, we describe a reverse-phase ion-pair HPLC assay coupled to absorbance and fluorescence detectors for the concurrent analysis of TP, 5-HT, 5-HIAA, T and IAA in samples of brain tissue.

EXPERIMENTAL

Chemicals: Solvents and reagents (analytical and chromatographic grades) were used as received from the supplier without further purification. L-tryptophan, serotonin hydrogen oxalate, tryptamine HCl, and 5-hydroxy-indole-3-acetic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Ion pair formation was performed by the addition of a 0.005 ml/L solution of 1-pentane sulfonic acid (PIC-B5) from Waters Associates (Milford, MA) to the mobile phase. Standard solutions of these TP metabolites were prepared by dissolving appropiate amounts in the same ratio than the initial conditions for HPLC assay, omitting the ion pairing reagent, and stored at 4°C.

Apparatus: The HPLC system consisted of two solvent delivery pumps (model 6000), a solvent programmer (model 660), a U6K injector, and a 30 cm long x 4 mm i.d. stainless steel column packed with μ-Bondapack C_{18} reverse phase, all from Waters. A guard column (also from Waters) was attached between the injector and the chromatographic column.

Column effluents were monitored with a Waters Model 441 Absorbance Detector at 280 nm, or with a 650-10 S Fluorescence Detector (Perkin-Elmer Co., Norwalk, CT) fitted with a 20 μL quartz flow cell for HPLC measurements. Fluorescence excitation and emission wavelengths were adjusted to 278

nm and 335 nm respectively, corresponding to the native fluorescence maxima of indole compounds. Slit widths for excitation and emission monochromators were selected to 5 and 10 nm respectively.

Chromatographic conditions: Low concentration eluent was a 0.005 mol/L solution of 1-pentane sulfonic acid (PIC-B5) at pH 3.0. High concentration eluent was a mixture of HPLC grade methanol and water (7/3, v/v). Both solvents were filtered and degassed before use. Different pH adjustements were achieved by the addition of formic acid or NaOH to the PIC-B5 solution. Chromatographic separation of TP metabolites were performed through a linear program (20% to 100% of the high concentration eluent, in 20 minutes) maintaining a flow rate of 1.0 mL/min. Column was held at room temperature.

Sample preparation: Adult Sprague-Dawley rats were sacrified by decapitation, their cortex and striata were removed as rapidly as possible, taken up in ice, blotted with filter paper, weighed and homogenized in 10 volumes of ice cold freshly prepared 0.1 mol/L HCl containing 1 mol/L KCl and 0.2% $NaHSO_3$(8). The homogenates were next centrifugated at 43000 x g for 20 minutes in a Sovall R5B centrifuge. The supernatants were then directly injected in the liquid chromatograph without any further extraction or purification step. Injection volumes ranged from 20 to 100 μL, corresponding to 2 to 10 mg of brain tissue.

RESULTS

In a previous study carried out in our laboratory (14) we described the separation of these TP metabolites with formic acid and water (1/1000, v/v) as a low concentration eluent. Now we have attempted to further im-

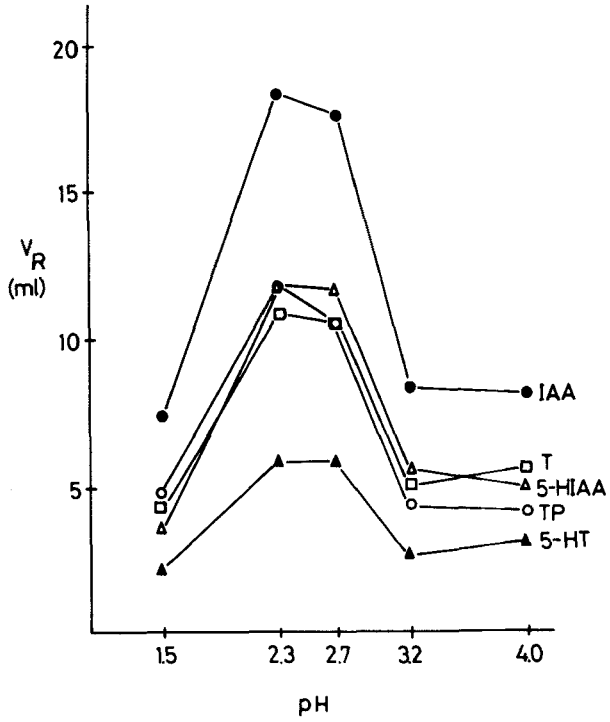

FIGURE 2. Dependence of the retention volumes of TP and its indole metabolites on the pH of the mobile phase. The chromatographic separation was achieved using HCOOH/H_2O (1/1000) as a low concentration eluent.

prove this separation by adjusting the pH of the mobile phase within the range of 1.5 to 4.0. The initial pH of the mixture of formic acid and water was 2.7. Lower and higher pH values than 2.7 were obtained by adding appropiate amounts of formic acid or NaOH respectively.

Figure 2 shows the dependence of the retention volumes of these metabolites on the pH of the mobile phase. As can be seen, T, 5-HIAA, and TP are only clearly re-

solved at pH 3.2 and 4.0 (pH 1.5 is avoided in routine analysis due to the damage caused to the column at such low pH). Higher pH values were not tested due to the better stability of indole compounds in acidic media (15). However, for quantitative purposes, such a separation was found not to be good enough, since T and 5-HIAA are only partially resolved at pH 3.2 (see Fig. 3). Even at pH 4.0, and in spite than the relative separation between 5-HIAA and T is better than that achieved at pH 3.2, there is not enough separation to allow for an adequate quantitation. Also, as shown in Figure 2, the resolution between these compounds cannot be improved drastically under these conditions since all of these compounds experience a similar degree of ΔV_R vs. ΔpH.

For these reasons, the formic acid in the mobile phase was substituted for an ion pairing reagent which acting as an ion suppressant increases the retention volumes of both amines, 5-HT and T. Accordingly the low concentration eluent was changed to a 5.10^{-3} mol/L solution of 1-pentane sulfonic acid (PIC-B5) at pH 3.1. The corresponding variations of retention volumes vs. pH values, like when formic acid, are plotted in Figure 4. As shown, the couple T and 5-HIAA is better resolved, and TP shows a different behavior pattern relative to the eluent prepared with HCOOH, probably due to its amphoteric properties which set it apart from the rest of the compounds. Here, the best separation is achieved at pH 2.7 (Figure 5) and 3.1, although other pH values could also be used depending on the nature of the sample. Thus, in brain tissue or CSF, in which T levels ($<$0.5 ng/g (8)) are not detectable using LC-Fluorometry, the pH could be $<$ 2.3, although values $<$ 2 are not recommended for column life.

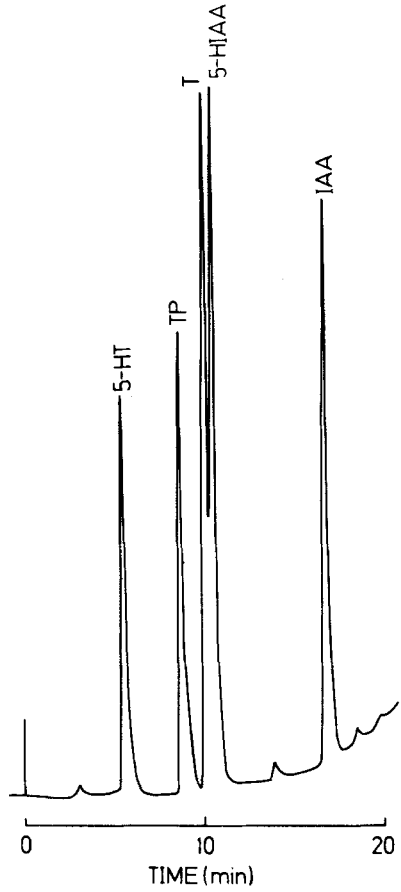

FIGURE 3. Chromatogram of a test mixture of TP metabolites obtained with HCOOH/H_2O at pH 3.2.

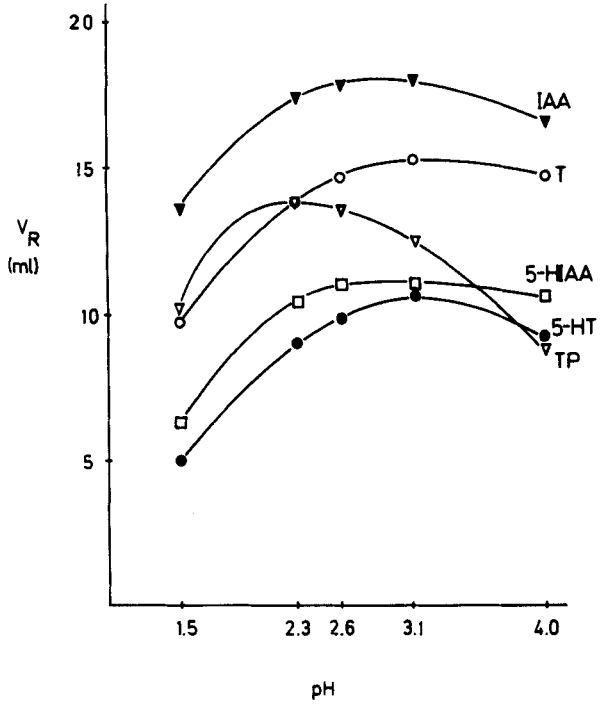

FIGURE 4. Effect of pH of mobile phase on retention volume values of TP and its indole metabolites using PIC-B5 as a low concentration eluent.

The detection limits of these indole compounds were measured using UV and fluorometric standard detectors. The values obtained are listed in Table 1. The response of all substances was found to be linear in the pg to μg range. The correlation coefficients of response curves are also listed in Table 1. No adsorption or degradation processes were found to occur for any of these indole compounds at the pg to ng level.

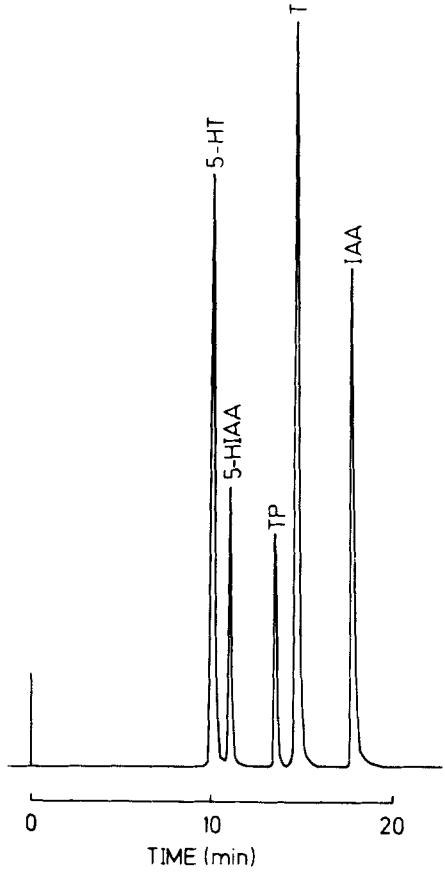

FIGURE 5. Chromatogram of a standard mixture of TP metabolites achieved with PIC-B5 at pH 2.7.

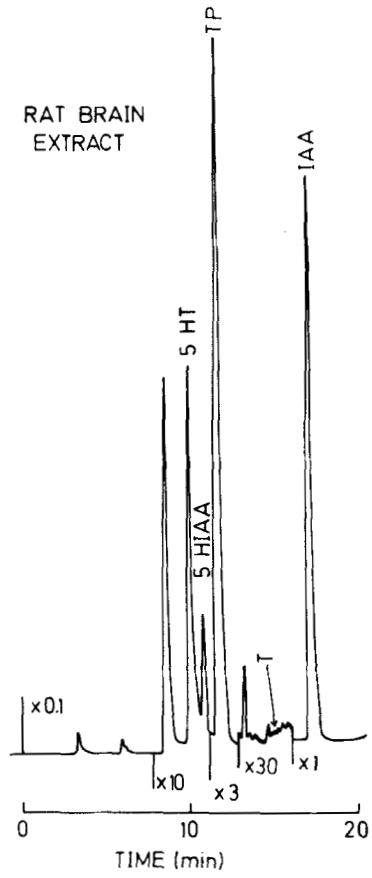

FIGURE 6. HPLC profile of a brain extract (cortex + striatum) of a rat treated i.p. with tryptamine (150 mg/Kg). Separation performed with PIC-B5 at pH 3.1 and monitored with a fluorescence detector (λ_{ex} = 278 nm, λ_{em} = 335 nm).

TABLE 1

DETECTION LIMITS AND CORRELATION COEFFICIENTS OF RESPONSE CURVES FOR TRYPTOPHAN AND ITS INDOLE RELATED METABOLITES

	ABSORBANCE	FLUORESCENCE	r^2 (*) pg-ng level	r^2 (*) ng-μg level
5-HT	5.36 ng	20 pg	0.9992	0.9997
5-HIAA	6.40 ng	120 pg	0.9985	0.9994
TP	4.16 ng	180 pg	0.9985	0.9989
T	2.47 ng.	30 pg	0.9978	0.9994
IAA	3.24 ng.	80 pg	0.9995	0.9999

(*) r^2 values correspond to response curves of fluorometric detection.

Figure 6 shows the injection of 100 μL of an homogenate (corresponding to 10 mg of tissue) of brain tissue of a rat treated i.p. with tryptamine (150mg/Kg) 30 minutes before death. Peaks of 5-HT, 5-HIAA, TP and IAA are cleary identified. Tryptamine is at concentration near the detection limits (20-30 ng/g of wet tissue) while IAA is in the order of 9 μg/g, thus indicating that most of the injected T has been rapidly metabolized to IAA through the action of monoamine oxidase (endogenous levels of IAA in control rats are in the order of 10-15 ng/g) (8,16).

DISCUSSION

The present work reports a simple and reliable HPLC system for the concurrent chromatographic separation of

TP and its four indole metabolites which optimizes the assay of indole compounds in a given sample. Two different low concentration eluent systems are reported: HCOOH/H_2O and 1-pentane sulfonic acid (PIC-B5)/H_2O, the later providing a better separation of the five substances studied. Nevertheless, due to low cost of the HCOOH/H_2O eluent, as compared to PIC-B5, it can still be recommended depending on the compounds of interest and the nature of the sample as mentioned above. Thus, if one is interested only in the metabolic pathway of the 5-hydroxyindoles (see Figure 1) the use of formic acid at pH 3.2 allows a direct estimation of the TP → 5-HT → 5-HIAA profile. In this case the exceedingly low amounts of tryptamine in body and tissue fluids would not interfere quantitatively with the 5-HIAA estimation. However, in samples containing similar amounts of all indole compounds, PIC-B5 would be preferred due to the better separation achieved.

Detection limits are in the order of 20 to 180 pg using an standard Perkin-Elmer 650-10 S Fluorescence Detector. For instance the detection limit of 5-HT (20 pg) allows the quantitation of this amine in minute amounts of rat brain tissue (less than 0.1 mg). Although the detection limits reported here for 5-HT and 5-HIAA are similar than those reported in the literature using electrochemical detection (12), this is not the case for TP, T and IAA, whose oxidation potentials are relatively high (17) so that the extra selectivity usually achieved with ECD is rapidly lost at the higher potentials due to the response of other interfering compounds. Thus for TP, T and IAA, fluorometry would still be the detection mode of choice in LC systems.

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REFERENCES

(1) Boullin, D.J. (Ed.) Serotonin in Mental Abnormalities. John Wiley & Sons. Chichester (1978).

(2) Warsh, J.J., Godse, D.D., Stancer, H.C. Chan, P.W. and Coscina, D.V. Biochem. Med. 18, 10 (1977).

(3) Martin, W.R. and Sloan, J.W. Psychopharmacologia 18, 231 (1970).

(4) Vogel, W.H. and Cohen, I. Brain Res. 122, 162 (1977).

(5) Jones, R.S.G. and Boulton, A.A. Life Sci. 27, 1849 (1980).

(6) Koch, D.D. and Kissinger, P.T. J. Chromatogr. (Biomed. Appl.) 164, 441 (1979).

(7) Saavedra, J.M., Brownstein, M. and Axelrod, J.J. Pharmacol. Exp. Ther. 186, 508 (1973).

(8) Artigas, F. and Gelpí, E. Anal. Biochem. 92, 233 (1979).

(9) Koch, D.D. and Kissinger, P.T. Life Sci. 26, 1099 (1980).

(10) Krstulovic, A.M., Brown, P.R., Rosie, D.M. and Champlin, P.B. Clin. Chem. 23, 1984 (1977).

(11) Anderson, G.M. and Purdy, W.C. Anal. Chem. 51, 283 (1979).

(12) Kempf, E. and Mandel, P. Anal. Biochem. 112, 223 (1981).

(13) Joseph, M.H., Kadam, B.V. and Risby, D. J. Chromatogr. 226, 361 (1981).

(14) Martínez, E., Tusell, J.M., Suñol, C., Mahy, N., Artigas, F. and Gelpí, E. Biological and Biomedical Applications of Liquid Chromatography IV. Hawk, G.L. (Ed.). Marcel Dekker. New York (1982).

(15) Geeraerts, F., Schimpfessel, L. and Crokaert, R. Clin. Chim. Acta 102, 247 (1980).

(16) Warsh, J.J., Chan, P.W., Godse, D.D., Coscina, D.V. and Stancer, H.C. J. Neurochem. 29, 955 (1977).

(17) Richards, D.A. J. Chromatogr. 175, 293 (1979).