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QUANTITATIVE DETERMINATION OF INDOLIC COMPOUNDS IN THE RAT BRAIN USING *p*-DIMETHYLAMINOCINNAMALDEHYDE AS REAGENT

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SUMMARY

A method is proposed for determining the metabolites of radioactive 5-hydroxytryptophan and of endogenous tryptophan in the rat brain. The compounds are separated by thin-layer chromatography and made visible with *p*-dimethylaminocinnamaldehyde (DACA) reagent. The conditions studied are those which allow the use of DACA reagent for the quantitative determination of tryptophan metabolites in brain tissue. Amounts of 5 ng of serotonin and 20 ng of tryptophan can be determined by densitometry.

INTRODUCTION

The indoleamine hypothesis of depression states that the level of serotonin (5-hydroxytryptamine, 5-HT) may be decreased in the brain of depressives. Therapy with the precursor tryptophan (Trp) has been attempted[1], as the concentration of Trp in the brain[2] is one of the factors that determines the synthesis of 5-HT. On the other hand, only a small part of Trp is converted into this amine. Thus, 5-hydroxytryptophan (5-HTP), an intermediate in the formation of serotonin, but not detectable in the brain, has been tried as a therapeutic agent for depression[3] and schizophrenia[4]. If radioactive serotonin is applied centrally (it does not cross the blood–brain barrier), it disappears from the brain within a few hours[5]. As the decarboxylation of 5-HTP is not the rate-limiting step of serotonin synthesis, it may be of importance to determine the metabolism of 5-HTP in view of its therapeutic value. The aim of this work was to develop a simple method for determining the metabolism of applied 5-HTP in the rat brain and its influence on endogenous serotonin metabolism.

Developing a thin-layer chromatographic (TLC) method for this purpose, the *p*-dimethylaminocinnamaldehyde (DACA) reagent of Harley-Mason and Archer[6] was used to stain the indolic compounds. In an earlier paper, the separation of some of the metabolites of Trp was reported[7]. In a semi-quantitative approach, Baumann

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and Narasimhachari[8] described the high sensitivity of the DACA reagent on cellulose but not on silica gel.

The use of the DACA reagent for the quantitative assay of Trp metabolites has not yet been proposed. The main purpose of this study was to test the highly sensitive DACA reagent as a tool for the quantitative measurement of tryptophan metabolites by TLC.

EXPERIMENTAL

Materials and reagents

The following equipment was used: scintillation counter, Packard Tri-Carb 3214; centrifuge, Sorvall RC-2; sample applicator, Waessle and Sandhoff prototype of the Linomat (Camag, Muttenz, Switzerland); electrically driven thin-layer plate coater, Camag; and densitometer, Zeiss (Oberkochen), complementary to the PMQ II. The compounds used were *p*-dimethylaminocinnamaldehyde (Schuchardt, Munich, G.F.R.), indolic compounds (Fluka, Buchs, Switzerland), microcrystalline cellulose (Avicel) (Merck, Darmstadt, G.F.R.) and cellulose MN 300 (Macherey, Nagel & Co., Düren, G.F.R.). Other reagents were Merck p.a. grade chemicals.

Radioactive compounds used were [3-^{14}C]D,L-5-hydroxytryptophan ([^{14}C]-5-HTP), 3.8 mCi/mmole (NEN, Boston, Mass., U.S.A.), [3-^{14}C]-5-hydroxytryptamine creatinine sulphate monohydrate ([^{14}C]-5-HT), 56 mCi/mmole (Radiochemical Centre, Amersham, Great Britain) and [1-^{14}C]-5-hydroxyindoleacetic acid ([^{14}C]-5-HIAA), 64 mCi/mmole (NEN, Boston, Mass., U.S.A.).

Method

In a typical experiment, 10 μl of a solution containing 58 μg (1 μCi) of [^{14}C]-5-HTP is injected intracisternally[5] in rats, with the aid of a 50-μl Hamilton syringe. Analyses of the endogenous compounds tryptophan and serotonin, and of the radioactive metabolites 5-HTP, serotonin and 5-HIAA, are performed on the whole brain or on parts of the brain after decapitation. The fresh brain samples are collected in ice-cold 0.4 *N* perchloric acid, then rinsed with 0.9% sodium chloride solution. One part of brain tissue is homogenised in 1.5 parts (w/v) of methanol. Alternatively, for small brain samples, the mixture is homogenised by sonication, by pressing the sound source on the outer wall of an Eppendorf reagent tube (1.8 ml) containing the mixture. As this procedure takes about 3 min, the sonication is performed in an ice-bath. The homogenates are centrifuged for half an hour at 31,000 *g* in the Sorvall centrifuge, then 100 μl extract are counted directly in a PPO–POPOP solution containing methanol. Extracts without radioactivity may occur if the intracisternal injection was unsuccessful; these extracts are not chromatographed.

Chromatography

An 18-g amount of Avicel cellulose is thoroughly mixed for 1 min with 100 ml of water with a ESGE mixer. This suspension is sufficient to coat seven thin-layer plates with the Camag coating apparatus. First, the 20 × 20 cm × 4 mm glass plates are cleaned with acetone so as to ensure the fixation of the 0.3 mm thick paste. The plates are dried in air.

In order to eliminate effects due to a possible uneven coating, the applications

of the mixture of standard substances and of the tissue samples are made alternately, with the Waessle and Sandhoff sample applicator. In order to obtain spots with the same width after the development, tissue samples are applied to a width of 26 mm and the standards to a width of 14 mm. On one plate, two 50-μl sample extracts together with three standard mixtures (15, 25 and 35 μl) of solutions containing, as a rule, 4 ng/μl of Trp, 4 ng/μl of 5-HTP and 1 ng/μl of 5-HT may be applied. The standards are dissolved in dilute methanol. The plates are developed in Desaga chromatography tanks with the following solvent mixture: butanone-2–acetone–2.5 *N* acetic acid (40:20:20). The running time for a distance of 12.5 cm is about 1.5 h. After the development, the plates are dried in air. The hR_F values already reported[7] are restated here: L-Trp, 50; 5-HT, 65; 5-HIAA, 98; and L-5-HTP, 35.

Densitometric measurement

A 2-g amount of DACA is dissolved in a mixture containing 100 ml of 6 *N* hydrochloric acid and 100 ml of ethanol. The reagent can be stored in a refrigerator for several weeks. The dry chromatograms are sprayed evenly with the reagent, but not until transparency occurs in order to avoid too high a background. Then, the plates are dried for 2 min at 60° in a drying cabinet. After this, the chromatograms are measured with the Zeiss densitometer, using the following settings: slit width of the monochromator, 0.06; slit length, 10 mm; wavelengths, 610 nm for blue spots (*e.g.*, serotonin) and 595 nm for violet spots (*e.g.*, Trp); scanning speed, 3; and recorder speed, 10.

The scanning proceeds in the same direction as the development of the chromatogram, after adjusting the baseline to $A = 0$. Each spot is scanned separately after first adjusting manually the point of maximal absorption of the spot. For quantitative evaluations, the peaks recorded were cut out and weighed. With the aid of the standard curve obtained, the values of the tissue samples could be determined, either mathematically or graphically.

Measurement of the radioactivity

For measurement of the radioactivity, Packard scintillation counting vials are filled with 15 ml of thixotropic gel, of which the background is determined. The thixotropic gel is prepared by dissolving 25 g of PPO and 1.5 g of dimethyl-POPOP in 5 l of pure toluene. To 1 l of this mixture, 40 g of thixotropic gel powder are added. After the densitometric measurements, the stained spots are scraped out and sucked into the vials under a light vacuum[9]. As the coloured spots may produce a high quenching, a quench curve must first be determined. For the measurement by the channel ratio method, the settings used are: windows, 50–1000 and 90–1000; gain, 15%. For carbon-14, recoveries of 40–80% are obtained under these conditions, depending on the quenching samples.

The results are given as mean values ± standard deviation.

RESULTS

Development of the method

Methanol was chosen as the solvent for homogenisation because it has a satisfactory volatility; acidic solvents would corrode the brass parts of the sample appli-

cator (Waessle and Sandhoff) and acidic solvents produce smearing of the spots on the chromatograms. As already reported[7], the proposed solvent system for TLC was the best for separating the most common indolic compounds in the rat brain. Urea (hR_F 57), giving a bright red spot with the DACA reagent, does not interfere under these conditions. Avicel cellulose was superior to MN 300 cellulose, as chromatography on the latter produces double spots for serotonin. In fact, the organic phase separates from the aqueous phase on the thin layer. The 5-HT spot is situated at the lower "front" (on top of the water layer) on Avicel cellulose. On MN 300, serotonin forms one spot on the organic and one on the water layer. Avicel cellulose also has the advantage of separating optical isomers. Fig. 1 shows the absorption spectra of 5-HT and Trp after staining the cellulose plates with DACA reagent. The spectra were obtained by measuring the absorption at discrete wavelengths at the point with the highest absorbance of the spot against the background. The uncorrected maxima, at a band width of 5 nm, are at 610 nm for serotonin and at 595 nm for Trp.

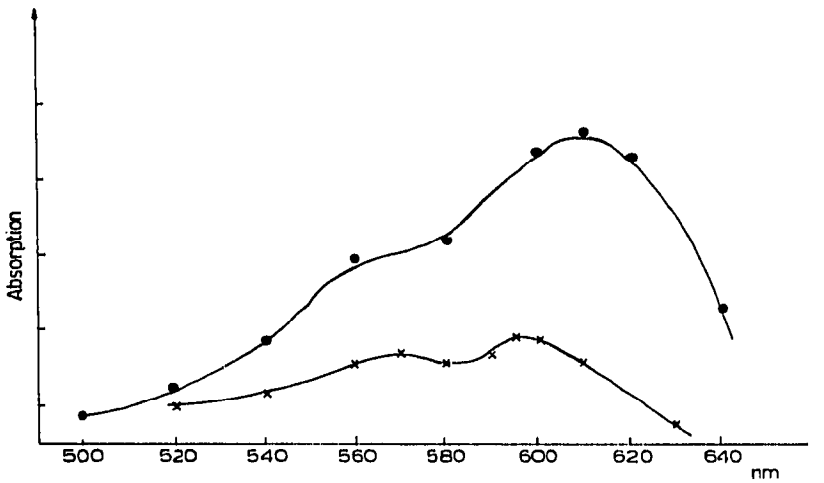

Fig. 1. Spectra of 5-HT (●) and Trp (×) after staining with DACA reagent on cellulose.

Harley-Mason and Archer[6] mentioned that the background may disturb the measurement. In Fig. 2, the results of four chromatograms, each with spots of 35 ng of serotonin, are shown. At the time 0 min, the plates are sprayed with DACA, then dried for 2 min with a hair-dryer. The first measurement occurred 3–4 min after the spraying procedure. This measured value was taken as 100% and other values were expressed as a percentage of this first value. During this experiment, the plates were kept in the dark, as light enhances the formation of a background. Fig. 2 shows that with increasing background the values decrease comparatively, the results differ from one plate to another, and the best time to measure the plates is between 10 and 30 min after staining.

The Zeiss densitometer allows measurements of both transmission and remission. In Fig. 3, these techniques are compared, showing standard curves for Trp and serotonin. It can be seen that 5 ng of 5-HT and 10 ng of Trp are still measurable. The sensitivity is higher in transmission than in remission, but the background is more stable in the latter, as in this case it is less dependent on the unevenness of the plates.

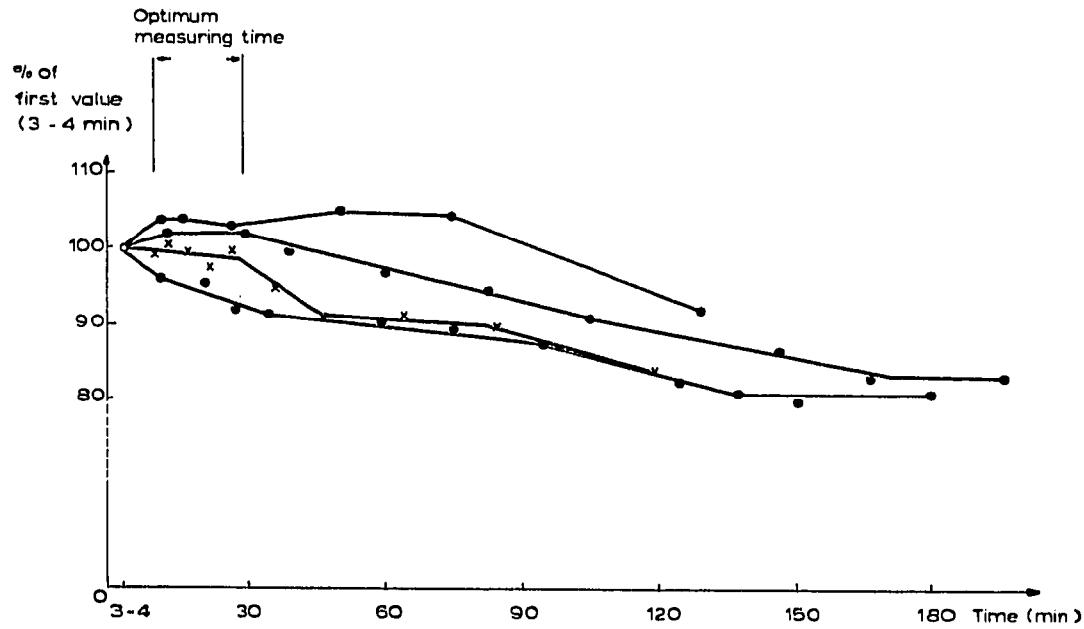

Fig. 2. Stability of the 5-HT–DACA reaction product as a function of time. Identification as in Fig. 1.

Of course, the sensitivity may still be augmented by applying smaller spots and closing the diaphragm. In this way, less uniform spots are obtained, giving less reproducible results and the Lambert–Beer law is no longer adhered to. This is clearly shown in an experiment where the same amount of 5-HT was applied as a band of 7 or 15 mm, and measured densitometrically after chromatography and detection with the DACA reagent with slit widths of 6, 10 and 14 mm (Fig. 4). As expected, the sensitivity can be increased by concentrating the substance on to a band of 7 mm and measuring with a slit width of 6 mm. However, at the same time, the reproducibility decreases, as shown by the widespread values of the three measured spots in this case. Therefore, final spots of 14 mm width, measured at a slit width of 10 mm, were preferred.

The blue spots and especially the reddish background formed by DACA produce a high quench. Thus, a cellulose plate was sprayed with DACA. To vials containing thixotropic gel and [^{14}C]-toluene increasing amounts of sprayed cellulose were added. By the ratio method, the quenching was established. The quench curve was used for the experiments, as the recovery may vary from 40 to 80%.

The recoveries of 5-HTP, 5-HT and 5-HIAA were estimated by adding the radioactively labelled compounds to four rat brains separately. After homogenisation and chromatography, the corresponding spots were scraped out and counted. The following recoveries were obtained: [^{14}C]-5-HTP, 91.4 $\pm$ 8.4%; [^{14}C]-5-HT, 68.9 $\pm$ 4.0%; [^{14}C]-5-HIAA, 79.1 $\pm$ 2.8%. For calculation, it was assumed that the brain has a density of unity and that the radioactive compound is equally distributed between the liquid and solid phases.

By this method, and by varying the extraction procedure and the solvent system for TLC, many indolic compounds could be determined quantitatively. Two examples are given here, *viz.*, the determination of 5-HT and Trp in the rat brain.

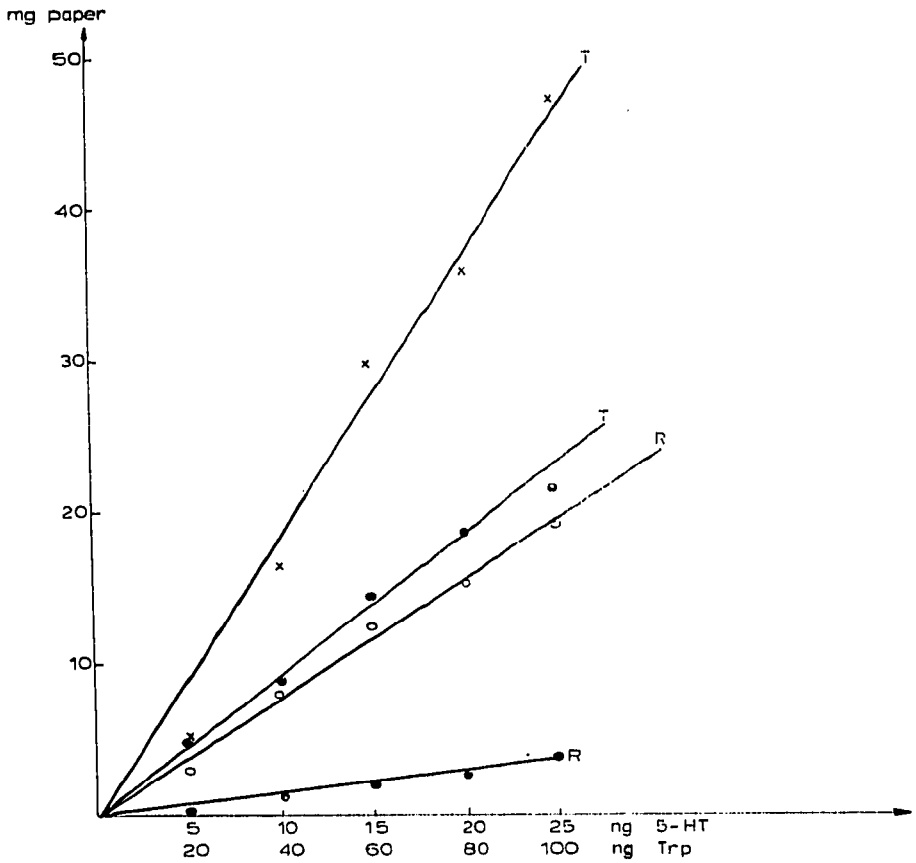

Fig. 3. Standard curves for 5-HT (at 610 nm) and Trp (at 595 nm) measured in transmission (T) and remission (R) applied as 14-mm spots on Avicel cellulose. (●), 5-HT; (○, ×), Trp.

For the determination of serotonin in the brainstem of Sprague-Dewley rats, three brainstems were pooled (mean weight 0.45 g) and homogenised in methanol. Eight double determinations on eight plates were carried out on the extract. On each plate, spots containing 15, 20 and 25 ng of serotonin as standards and two spots of 75 μl of the extract were applied. Fig. 5 shows the mean standard curve for the eight plates and the mean value of the eight double determinations of the tissue extract. The calculated value is 28.4 $\pm$ 3.8 ng (13%) per 75-μl extract. Considering the recovery, the 5-HT content of the brainstem is 1340 $\pm$ 175 ng per gram of tissue. For the determination of Trp in the brainstem, six double determinations on six plates were carried out, by applying 60, 100 and 140 ng of Trp and twice 50 μl of the supernatant. Table I gives the measured values. The recovery is 5.35 $\pm$ 0.33 μg of Trp per gram of tissue.

Note: the considerable variations in the absolute values from one plate to another indicate the necessity for comparing standards on the same plate.

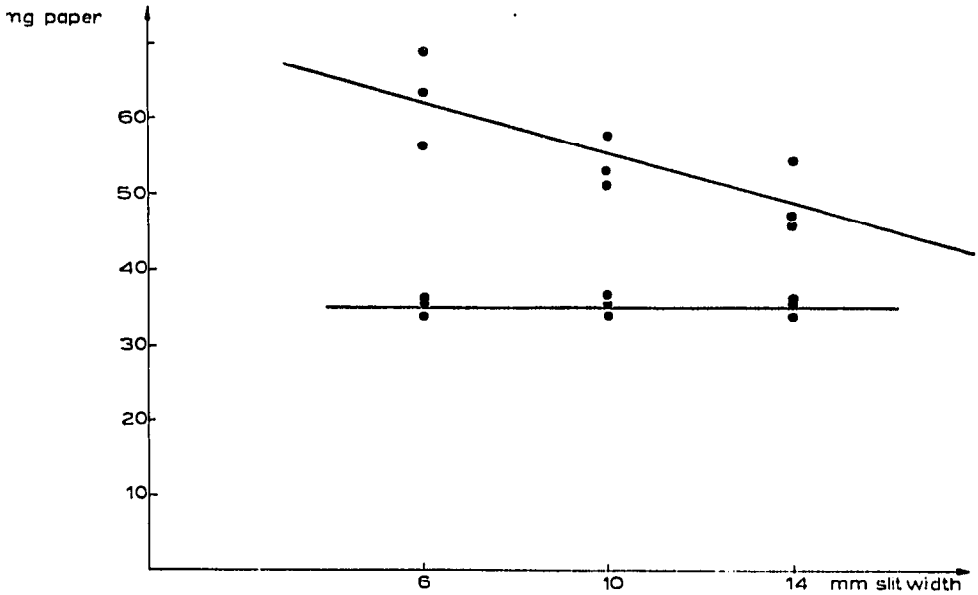

Fig. 4. Dependence of the measured value (expressed as milligrams of paper) on the slit width and the width of the applied spot. The same amount of serotonin was applied as a band of 7 mm (upper line) and of 15 mm (lower line).

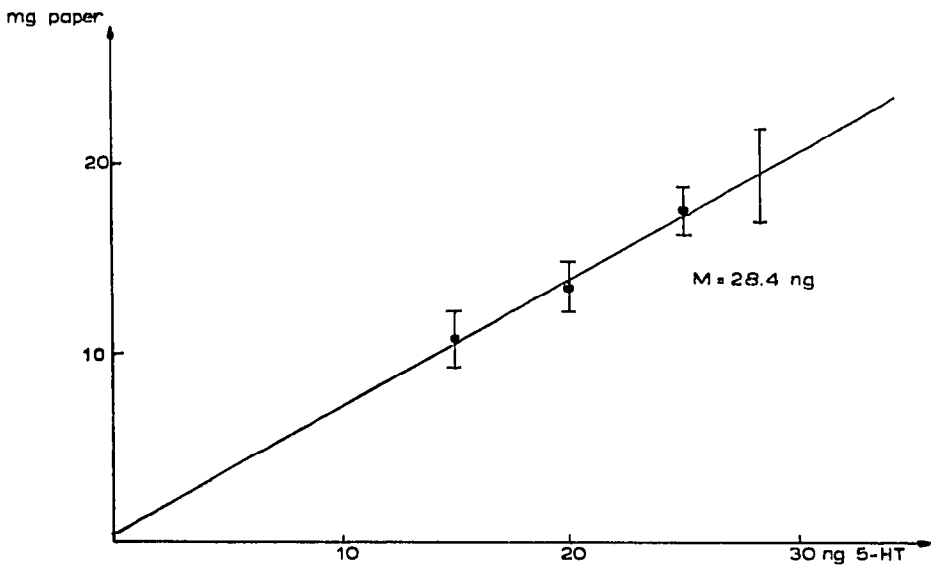

Fig. 5. Determination of 5-HT in three pooled brainstems of rats. The standard curve and the tissue value are means of eight double determinations. The standard curve is derived from 5-HT spots of 15, 20 and 25 ng.

TABLE I

DETERMINATION OF TRYPTOPHAN IN FOUR POOLED BRAINSTEMS OF RATS

The measured values are expressed as mg "peak weight".

Chromatogram	*Measured values of the standard curve*			*Mean value of 2 × 50-µl brain extract*	*Mean values (ng)*
	60 ng	*100 ng*	*140 ng*		
1	8.7	26.2	59.4	25	90
2	11.5	13.4*	27.8	16.2	86
3	17.4	25.2	33.0	21.8	82
4	24.1	49.0	68.4	37.2	80
5	18.2	41.3	72.9	28.8	77
6	16.9	—	62.6	26.4	78
					Mean: 82 ± 5 ng per 50 µl extract

* 80 ng.

Application

This method permits a pharmacokinetic study to be made of the metabolism of 5-HTP, serotonin and 5-HIAA and the influence of drugs upon it.

In an experiment, one group of five Sprague-Dawley rats was not pre-treated, while another group of six animals received an intracisternal injection of 58 µg of [^{14}C]-5-HTP (1 µCi). One hour later, the eleven rats were decapitated and assayed for [^{14}C]-5-HTP, [^{14}C]-5-HT, [^{14}C]-5-HIAA, 5-HTP, Trp and 5-HT. Fig. 6 shows a typical chromatogram from this experiment and its densitometric measurement. The left-hand curve replicates the measurement of the first line, with the following standards:

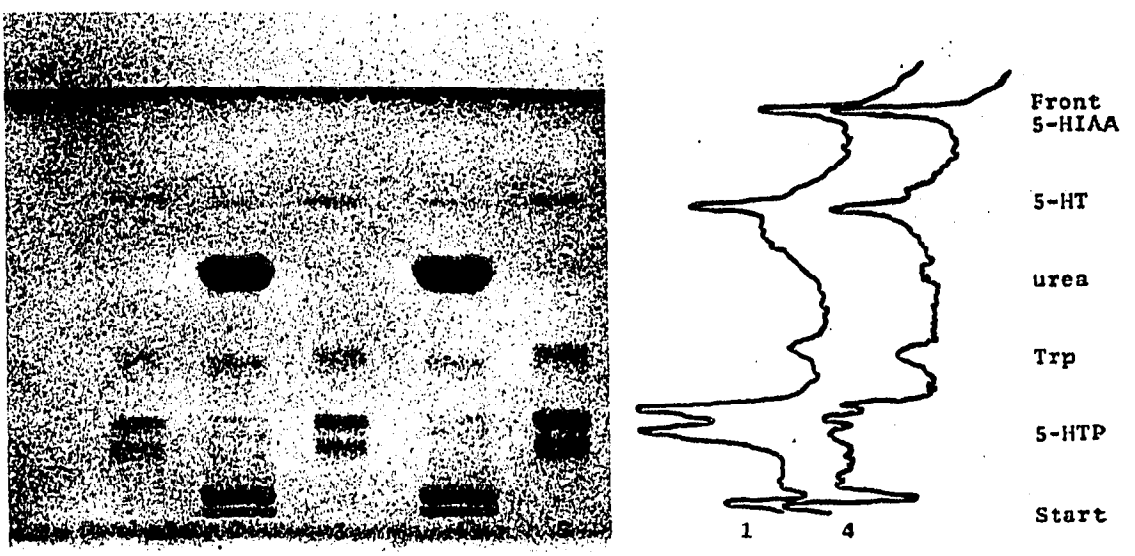

Fig. 6. Chromatogram and graphs of its densitometric measurement in transmission of a brain tissue extract and standards.

5-HTP (60 ng), 5-HT (15 ng) and Trp (60 ng). The good separation of the optical isomers of 5-HTP can be seen; in isolated experiments with the D- and L-forms of 5-HTP, it appeared that the D-form moves faster. This clear separation could not have been achieved when tissue extracts were chromatographed: the right-hand curve shows the measurement of line 4. As the recording was carried out at 610 nm, the bright red spot of urea was filtered out almost quantitatively. In the two curves, the high background at lower R_F values than that of 5-HT is striking, and may be due to water-soluble impurities in the cellulose. Results are given in Table II.

In untreated animals, 5-HTP is not detectable, which is in agreement with previous work[10]. The radioactive 5-HTP is still measurable densitometrically 1 h after its injection. In the last columns, the proportion of each radioactive compound is expressed as a percentage of the total remaining radioactivity. 5-HTP remains predominant. The newly formed 5-HT is rapidly metabolised, which is shown by its relatively small proportion. By application of [^{14}C]-5-HTP, the total 5-HT does not increase in comparison with the value in untreated animals.

DISCUSSION

Several papers have described the separation of indolic compounds on silica gel[11–23], but only Contractor and Wragg[24] studied the resolution of Trp metabolites on cellulose. Applications of the quantitative assay of indolic compounds by densitometry are rare[12,18]. Although the DACA reagent has been widely used as a spray reagent for qualitative measurements[6,8,20,22], no attempt has been made to prove its use in the quantitative determination of indolic derivatives by densitometry.

By densitometry (Fig. 3), the visual impression of the high sensitivity of the reagent has been confirmed. Standard curves on a single chromatogram show that linearity exists for the ranges 5–25 ng of 5-HT and 20–100 ng of Trp. On the other hand, all experiments failed to obtain comparable standard curves from one plate to another. Fig. 2 shows that the stability of the product may vary from one plate to another. In this regard, the DACA reagent is less advantageous than 4-dimethylbenzaldehyde reagent, where the reaction products are relatively stable[12]. As is clearly shown by Table I, it is strongly recommended that standards are carried on the same plate as the extract. However, as the DACA reagent is about ten times more sensitive than Van Urk's reagent, it may be useful for problems where a high sensitivity is especially needed, such as for measurements of indoles in brain samples. In this respect, it may be compared with the OPT reagent[8]. Nevertheless, after TLC, the OPT reaction products have only been determined after extraction of the silica gel[16,17] and never by densitometry. Paraformaldehyde could be an equally sensitive reagent as DACA, as it gives a fluorescent product with serotonin at the nanogram level[11]. Until now, no application with paraformaldehyde reagent has been reported.

It is noteworthy that, as the reaction with DACA takes place at the C-2 position of the indole ring, the composition of the C-3 side-chain does not determine the colour formation among the tested compounds. More important is the C-5 position, because if this position is not occupied a violet colour is produced (DMT, tryptamine, Trp, indoleacetic acid). A hydroxy or methoxy group at the C-5 position is responsible for a higher reaction rate, which may be explained by the positive mesomeric effect caused by the oxygen. This appears as a more intense blue colour after

TABLE II

FATE OF 58 μg (1 Ci) OF [^{14}C]-5-HTP IN THE RAT BRAIN AFTER INTRACISTERNAL INJECTION AND ITS INFLUENCE ON THE ENDOGENOUS TRYPTOPHAN METABOLISM

Group	*5-HTP*			*5-HT*			*Trp* (*ng/g tissue*)	*5-HIAA* (*dpm/g tissue*)	*Percentage of total radioactivity*		
	ng/g tissue	*dpm/g tissue*	*dpm/ng*	*ng/g tissue*	*dpm/g tissue*	*dpm/ng*			*5-HTP*	*5-HT*	*5-HIAA*
Controls (5 animals)	–	–	–	1171±188	–	–	3880±860	–	–	–	–
[^{14}C]-5-HTP-treated (6 animals)	1840±350	135500±38500	73±13	1180±300	6125±540	5.4±1.7	4500±1100	22500±2000	82.47±1.63	3.74±0.21	13.79±1.53

spraying serotonin, 5-hydroxytryptophan, 5-methoxy-N,N-dimethyltryptamine, etc., with the DACA reagent. The reaction may be similar to that described by Dibbern and Rochelmeyer[25] for the Van Urk reagent.

The extraction procedure with methanol–water is not very specific but permits the extraction in a single step of all of the substances tested. Other compounds that may interfere on the chromatogram occur in the brain in very low concentrations. Melatonin, highly concentrated in the pineal gland[26], is not synthesized in the remainder of the brain. Chase *et al.*[27] injected serotonin intracisternally. The neutral metabolites (5-hydroxytryptophol?) formed were only about 7% of the mean metabolite 5-HIAA. Information about 5-hydroxytryptophol in the rat brain is scarce. Klein and Notides[28] showed *in vitro* that after 24 h the principal pineal gland derivative formed from serotonin is 5-HIAA. The 5-hydroxytryptophol formed was only about 1.5% of the incubated serotonin.

By the present method, 1340 and 5350 ng/g of 5-HT and Trp, respectively, were measured in the brainstem. Maickel *et al.*[29], Curzon and Green[30] and Shellenberger and Gordon[31] found 1230, 1030 and 941 ng/g of serotonin, respectively, in the midbrain. Tagliamonte *et al.*[32] showed the Trp level in the rat brain to be 4.56 μg per gram of tissue.

The application with [^{14}C]-5-HTP presented here is one of a series in which the disappearance of radioactive 5-HTP and its metabolites as a function of time will be studied. However, it already appears that serotonin is not accumulated after intracisternal injection of [^{14}C]-5-HTP, but is rapidly metabolised to 5-HIAA.

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