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NEW GAS CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF 5-HYDROXYTRYPTAMINE AND 5-HYDROXYINDOLEACETIC ACID IN THE BRAIN*

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Abstract—A new procedure for the extraction of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) and a very sensitive gas chromatographic method for the analysis of these compounds are described. 5-HT and 5-HIAA are extracted from the brain into 20% butanol in ether. The extracted compounds are silylated with trimethylsilylimidazole and analyzed gas chromatographically on a column filled with solid gaschrom-Q, 80–100 mesh, which was coated with 3% OV-17. The method can be used over a wide range of concentrations, and amounts as little as 2·5 ng can be accurately measured. Drugs that lower or elevate the brain concentrations of 5-HT and 5-HIAA were used to test the applicability of this method. The adaptation of the extraction procedure to allow the analysis of 5-HT and 5-HIAA by filter fluorometry is also described.

GAS CHROMATOGRAPHIC methods have been reported for several biologically important amines including 5-hydroxytryptamine (5-HT)[1, 2] and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA).[3] However, a method for the gas chromatographic analysis of 5-HT and 5-HIAA from biological samples has not yet been established. Previously, when these highly polar compounds were extracted from tissue, it was difficult to derivatize them by silylation so that a single derivative could be obtained. A few years ago, Horning *et al.*[4] succeeded in obtaining single derivatives of catecholamines by silylating with trimethylsilylimidazole (TMS-imidazole) which specifically silylates hydroxyl groups. The present report describes in detail a method to obtain a single peak of both 5-HT and 5-HIAA on the gas chromatogram using TMS-imidazole to make the derivatives. A new extraction procedure for these compounds, the adaptation of this extraction procedure for fluorometric analysis, and the application of these methods are also included.

MATERIALS AND METHODS

Chemicals. All the common laboratory chemicals employed were reagent grade obtained from either the J. T. Baker Chemical Co. or the Mallinckrodt Chemical Works. *n*-Butanol was bought from Merck & Co. Ninhydrin and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine were bought from the Aldrich Chemical Co. Trimethylsilylimidazole (TMS-imidazole) was obtained from the Pierce Chemical Co. 5-HT and 5-HIAA were obtained from the Sigma Chemical Co.

Animals. Male Swiss–Webster mice (Simonsen) weighing 25–30 g were used in all the experiments.

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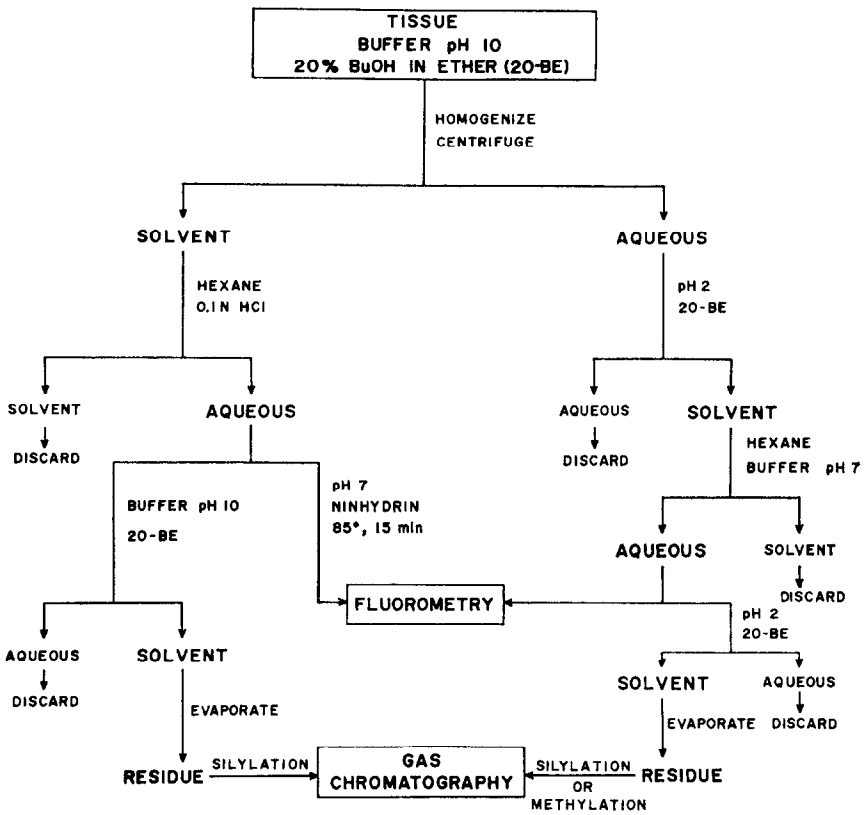

FIG. 1. Flow sheet of the procedure for the extraction of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid from brain tissue. 5-HT is contained in the initial solvent phase and 5-HIAA is contained in the initial aqueous phase. Details of the steps are described in the Method section.

Extraction from tissue. The entire extraction procedure is outlined in Fig. 1. Five ml of 20% butanol in ether* (20-BE) and 1 ml of 0·75 M phosphate buffer, pH 10 (saturated with NaCl), were added to a small homogenizing tube (No. 4288-C Grinding Vessel, Arthur H. Thomas Co.) containing either one-half (140–240 mg) or a whole brain (380–480 mg) from one mouse, and the mixture was homogenized. The homogenizing tubes were then centrifuged at 3000 rev/min for 5 min. For the extraction of 5-HT, 4 ml of the clear solvent phase was transferred to a small test tube and evaporated to approximately 1 ml with the aid of a water bath at 60° and a gentle flow of air. Next, 1·5 ml of 0·1 N HCl and 5 ml of hexane were added to the tube. The tube was sealed with a caplug (Protective Closures Co., Inc.), shaken for 5 min and centrifuged at 3000 rev/min for 5 min. One ml of the pH 10 phosphate buffer was added to 1 ml of the aqueous phase and re-extracted with 2 ml of 20-BE. The upper solvent phase was quantitatively transferred to a small test tube with a Pasteur pipette and evaporated to dryness with a gentle flow of air. The residue was used for

* Ether used in this study was anhydrous diethyl ether, analytical reagent grade and will be referred to in the text simply as ether.

silylation and analysis by gas chromatography. The total recovery of 5-HT was 51·4 ± 2·7 per cent, uncorrected, and 91·0 ± 4·7 per cent corrected, for four determinations.

For the extraction of 5-HIAA, the aqueous phase from the initial extraction, including the tissue at the interface and 1 ml of the remaining solvent phase, was adjusted to pH 2 with 3 N HCl and extracted with an additional 4 ml of 20-BE. Four ml of the solvent phase was evaporated to approximately 1 ml, shaken with 5 ml of hexane and 1·5 ml of 0·05 M phosphate buffer, pH 7, and centrifuged. One ml of the aqueous phase was then adjusted to pH 2 with 1 N HCl and extracted with 2 ml of 20-BE. The solvent phase was evaporated to dryness and the residue was used for silylation and analysis by gas chromatography. The total recovery of 5-HIAA was 53·3 ± 2·2 per cent, uncorrected and 90·7 ± 3·8 per cent, corrected, for four determinations.

Silylation procedure. For the silylation of 5-HT, the residue from the extraction was dissolved in 50 μl of 1:1 mixture of TMS-imidazole–acetonitrile and the tube was sealed with a caplug and heated for 2 hr in a water bath at 60°. The silylation of 5-HIAA was the same except that only 1 hr heating time was sufficient. 5-HIAA can also be esterified with an ether solution of diazomethane;[5] however, the silylation procedure is preferable.

Gas chromatography. A Varian 1200 equipped with a flame ionizing detector was used. The column consisted of a glass column (6 mm O.D., 3 mm I.D.) 6 in. long at the injection portion which was connected to a coiled stainless steel tube (0·125 in. O.D., 0·085 in. I.D.) 6 ft long. The column was filled with solid gaschrom-Q, 80–100 mesh, which was coated with 3% OV-17 (Applied Science Laboratories, Inc.). For the analysis of 5-HT, the temperature of the column was 210°, that of the injection portion was 250° and that of the detector was 270°. For the analysis of 5-HIAA the temperature of the detector was the same as that for 5-HT; however, that of the injection portion was 180° and that of the column was 150°.

Adaptation for fluorometric analysis. At appropriate steps in the extraction outline presented in Fig. 1, one can use this procedure for the analysis of 5-HT and 5-HIAA by fluorometry. Fluorescence because of 5-HT was made more specific by the use of ninhydrin as described by Vanable.[6] One ml of the appropriate aqueous phase (Fig. 1) was placed in a test tube containing 1 ml of 0·75 M phosphate buffer, pH 7, and mixed. An aqueous ninhydrin solution (0·1 M) was made just before use, and 0·1 ml of this solution was added to the above tube and the tube was heated in a water bath at 85° for 15 min. After heating, the tubes were left at room temperature for 1 hr. The volume was then diluted to 4 ml with distilled water and the fluorescence was determined in a filter fluorometer. The recovery of 5-HT from tissue was 93·3 per cent corrected.

For the analysis of 5-HIAA, 1 ml of the appropriate buffer phase (Fig. 1) was simply diluted to 4 ml with distilled water and fluorescence was determined on the filter fluorometer. The recovery of 5-HIAA from tissue was 98·0 per cent corrected.

Filter fluorometry. Fluorescence was determined on a Model 111 Turner Fluorometer equipped with a far ultra-violet lamp (General Electric No. G4T4-1). The primary filter used was No. 7–54 (Corning). The secondary filter was No. 3 (Kodak-Wratten) when assaying for 5-HT and was No. 47-B (Kodak-Wratten) when assaying for 5-HIAA. It is important to note that all measurements must be made in quartz tubes.

RESULTS AND DISCUSSION

Effects of various organic solvents on the extraction of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid. The percentage extraction of authentic 5-HT and 5-HIAA by several organic solvents from aqueous solutions of pH 2 and pH 10 buffers is shown in Table 1. The best extraction of the two compounds was accomplished with 20% butanol in ether (20-BE) and this solvent was better than the commonly used

TABLE 1. EXTRACTION OF 5-HYDROXYTRYPTAMINE AND 5-HYDROXYINDOLEACETIC ACID FROM BUFFER USING SEVERAL ORGANIC SOLVENTS

Solvents	Mean % extracted by solvent ± S.E.*			
	pH 2·0†		pH 10·0‡	
	5-HT	5-HIAA	5-HT	5-HIAA
20% Butanol in ether	7·1 ± 0·8	99·9 ± 0 1	98·2 ± 0·5	0
10% Butanol in ether	5·0 ± 0·6	98·9 ± 0·5	73·6 ± 1·4	0
Ether	4·7 ± 0·6	66·7 ± 6·6	29·5 ± 2·5	0
20% Butanol in chloroform	2·5 ± 0·4	44·9 ± 3·0	63·2 ± 1·2	0
20% Butanol in ethyl acetate	12·0 ± 1·4	85·4 ± 3·8	84·5 ± 2·3	0
10% Butanol in ethyl acetate	8·5 ± 0·9	82·0 ± 2·7	75·0 ± 2·4	0
20% Butanol in benzene	8·2 ± 0·7	51·0 ± 3·1	45·5 ± 2·5	0
Butanol	76·0 ± 6·2	93·3 ± 3·5	84·0 ± 2·3	0

* M ± S.E. from four determinations.
† 0·05 M Oxalic acid–NaOH buffer, pH 2·0, saturated with NaCl.
‡ 0·75 M Na_2HPO_4–NaOH buffer, pH 10·0, saturated with NaCl.

butanol.[7–9] A single extraction by 20-BE gave 98·2 per cent recovery of 5-HT and 100 per cent recovery of 5-HIAA, whereas recoveries using butanol were 84 per cent for 5-HT and 93·3 per cent for 5-HIAA. Recoveries using various combinations of butanol with chloroform, ethylacetate or benzene were not as good when compared to the above two solvents. 20-BE proved to be a very good solvent not only for its excellent extraction characteristics but also for the ease in which it can be evaporated so that samples can be prepared quickly and easily for gas chromatographic analysis.

Sensitivity and standard curves. Sharp, single peaks of 5-HT and 5-HIAA were observed on the gas chromatogram when the compounds were silylated with TMS-imidazole. There was an excellent positive correlation between peak heights and the amount of the compounds. The sensitivity of the method and examples of standard curves of silylated 5-HT and 5-HIAA are shown in Fig. 2. The peak heights on the gas chromatogram were clearly proportional to the amounts of the compounds and amounts as low as 2·5 ng could be easily quantitated.

Recovery of tissue. The efficiency of the assays described in the Method section was tested by studying the recoveries of added 5-HT and 5-HIAA to brain tissue. The results of these experiments are shown in Fig. 3. The recovery peaks shown for 5-HT and 5-HIAA represent a 50 ng added standard plus the endogenous amounts of the compounds extracted from a single mouse brain. When the added standard was subtracted from these peaks, the endogenous concentration of 5-HT was estimated to be 0·55 μg/g tissue and that of 5-HIAA was 0·22 μg/g tissue.

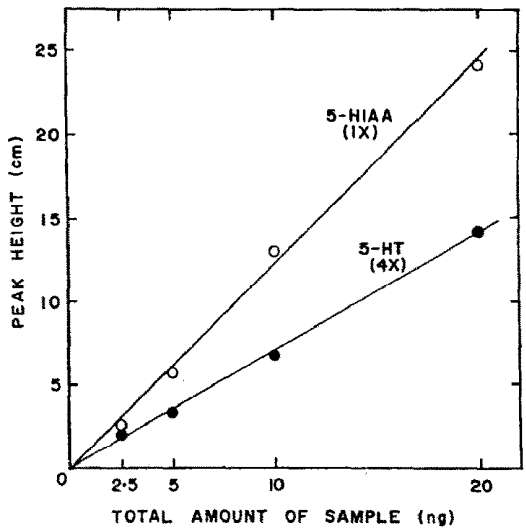

FIG. 2. Standard curves for the analysis of silylated 5-hydroxytryptamine and 5-hydroxyindoleacetic acid by gas chromatography. The compounds were silylated with TMS-imidazole as described in the Method section. The numbers in parentheses are relative sensitivity settings on the gas chromatograph. The determination of 5-HT was performed at four times the sensitivity setting used for the determination of 5-HIAA.

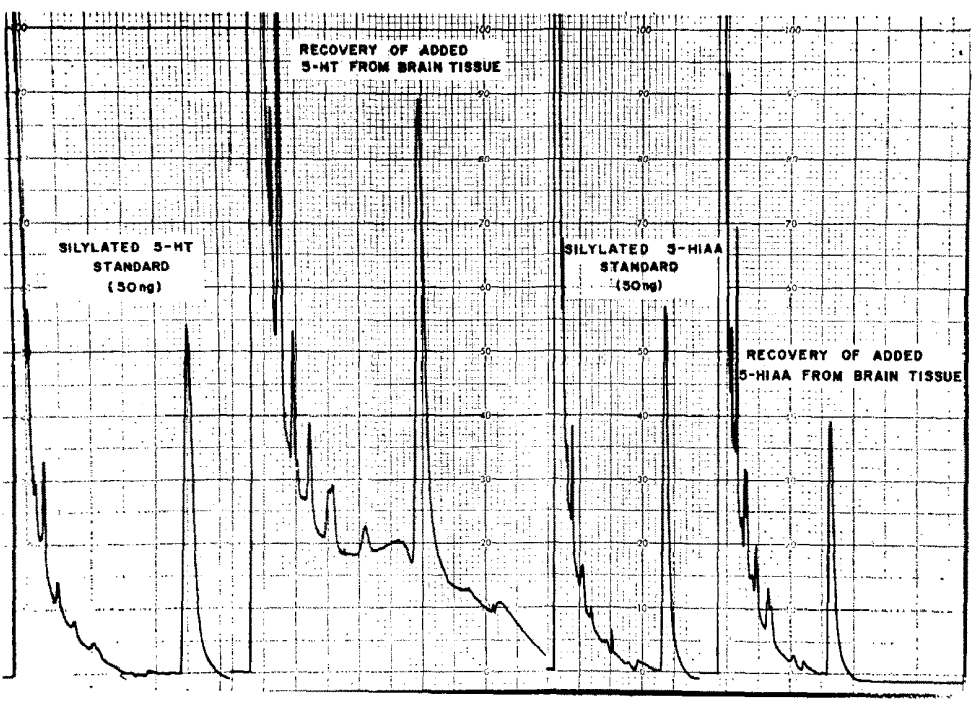

FIG. 3. Recovery of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid added to brain tissue. Both compounds, 50 ng each, were added to a single mouse brain and extracted in the manner shown in Fig. 1. Compounds were silylated with TMS-imidazole. The retention times for 5-HT and 5-HIAA were 13 min and 17 min respectively.

Fluorometry. The extraction procedure outlined in Fig. 1 can be adapted for use with a fluorometric assay as indicated in the Method section. Standard curves for the determination of 5-HT and 5-HIAA are shown in Fig. 4. The different curves represent different concentration ranges and different sensitivity settings on the Turner fluorometer. The assays of both 5-HT and 5-HIAA were linear between 1·0 and 250 ng/ml. The sensitivity was a little less than the gas chromatographic method, however, it was possible to assay amounts as little as 4 ng of 5-HT or 5-HIAA by the extraction procedure described herein.

Effect of p-*chlorophenylalanine (PCPA) and pargyline.* The applicability of the present methods was tested by studying the known effects of PCPA,[10] a tryptophan hydroxylase inhibitor, and pargyline,[11] a monoamine oxidase inhibitor, on the endogenous amounts of 5-HT. A typical experiment is shown in Fig. 5. The endogenous amount

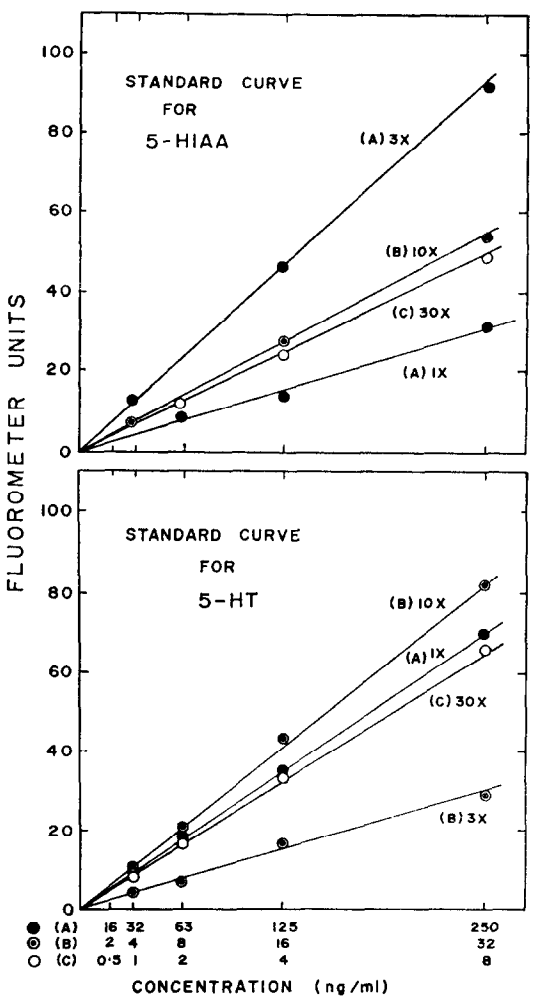

FIG. 4. Standard curves for the fluorometric determination of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid. The various curves represent different concentration ranges and different sensitivity settings on the filter fluorometer.

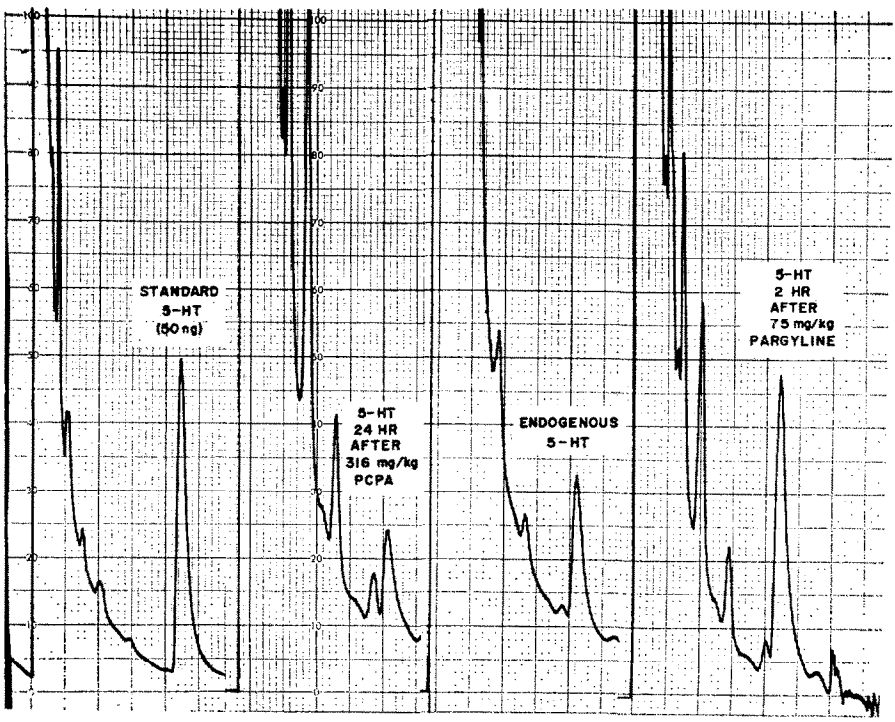

FIG. 5. Effect of *p*-chlorophenylalanine (PCPA) and pargyline on the brain concentration of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid. The compounds were silylated with TMS-imidazole. The third peak represents the actual amount of endogenous 5-HT without added standard.

of 5-HT without added standard is shown for comparison. The endogenous amounts of 5-HT fell after treatment with PCPA and increased after treatment with pargyline as expected. This experiment was repeated with several animals and the quantitation by gas chromatography of both 5-HT and 5-HIAA was compared to that by filter fluorometry on the same samples. The results of this comparison are shown in Table 2. The normal endogenous concentrations of 5-HT and 5-HIAA were similar to those reported by other investigators.[8, 12–16] Results similar to those described above were obtained after treatment with PCPA or pargyline. In addition, treatment of both drugs caused a decrease in the concentration of 5-HIAA. The values obtained with the gas chromatographic analysis and those obtained with the fluorometric analysis were very comparable and there were no significant differences between the results of the two methods.

It must be pointed out that one of the disadvantages of the fluorometric method is that the contribution of the nonspecific fluorescence of the tissue blank cannot be accurately determined and may result in increased fluorescent readings. This is especially true when a filter instrument is being used. Thus, in this study the tissue-blank fluorescence was initially established with the aid of the gas chromatographic method. The tissue-blank fluorescence represented 10 per cent of the total fluorescence observed on the filter fluorometer. The results reported in Table 2 have been corrected for this nonspecific tissue fluorescence.

TABLE 2. EFFECT OF *p*-CHLOROPHENYLALANINE (PCPA) AND PARGYLINE ON BRAIN CONCENTRATIONS OF 5-HYDROXYTRYPTAMINE AND 5-HYDROXYINDOLEACETIC ACID*

Treatment	μg/g Wet weight			
	5-HT		5-HIAA	
	Gas chromatography	Fluorometry	Gas chromatography	Fluorometry
Control	0·51 ± 0·06	0·52 ± 0·04	0·18 ± 0·01	0·20 ± 0·01
Pargyline 75 mg/kg i.p. 2 hr before sacrifice	1·35 ± 0·03	1·23 ± 0·05	0·06 ± 0·02	0·10 ± 0·02
PCPA 316 mg/kg i.p. 24 hr before sacrifice	0·27 ± 0·03	0·24 ± 0·02	0·05 ± 0·01	0·06 ± 0·01

* Values represent M ± S.E. from four mice.

Shortly after the present study was finished, a simpler silylating procedure was discovered. The final residue from the extraction is dissolved in 50 μl of a 1:1 mixture of TMS-imidazole and acetonitrile. The solution is simply shaken well and injected into the gas chromatograph column. This procedure avoids the heating for 2 hr at 60°. An additional time saving feature is that the retention time of the peak because of 5-HT is only 3·5 min. The nature of this derivative is presently being investigated by mass spectrometry. The new procedure, however, is not as sensitive and particular care must be used to construct the standard curve because it is not quite linear. The new procedure can be used to estimate amounts of 5-HT down to 10 ng, and endogenous amounts of 5-HT from mouse brain can be determined readily.

In summary, we have described a new procedure for the extraction of 5-HT and 5-HIAA from the brain and have established a specific, sensitive and accurate gas chromatographic method for the analysis of these compounds. We have also described how the extraction procedure could be adapted for use in the analysis of these compounds by filter fluorometry.

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