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MASS FRAGMENTOGRAPHIC DETERMINATION OF 5-HYDROXYTRYPTAMINE AND 5-HYDROXYINDOLE-3-ACETIC ACID IN BRAIN TISSUE USING DEUTERATED INTERNAL STANDARDS

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SUMMARY

A mass fragmentographic method for the determination of 5-hydroxytryptamine (5-HT) and 5-hydroxyindole-3-acetic acid (5-HIAA) in the same extract of rat brain tissue is described. Deuterium-labelled analogues were used as internal standards. 5-HT and 5-HIAA were separated by solvent extraction and pentafluoropropionyl derivatives were prepared for the mass fragmentographic analysis. Multiple ion analysis confirmed the identity of 5-HT and 5-HIAA in the rat brain. At the mass numbers used routinely for the determination of 5-HT and 5-HIAA, the experimental error was below 3% (calculated from mean values of 0.50 and 0.24 nmole, respectively). The recovery of the authentic compounds added to brain extracts was more than 95%. The levels of 5-HT and 5-HIAA in the rat brain were 2.95 $\pm$ 0.16 and 0.64 $\pm$ 0.18 nmole/g, respectively. More than 100 samples could be analyzed within 3 days. The presence of 5-hydroxytryptophol in rat brain was also investigated, but none could be detected either as a conjugate or as the free alcohol.

INTRODUCTION

The metabolism of 5-hydroxytryptamine (5-HT) in the brain has been of interest since the discovery that antidepressant and psychotomimetic drugs affect its turnover. For the further elucidation of the mechanism of action of these drugs, the development of a specific and highly sensitive mass fragmentographic method for the determination of 5-HT and its main metabolite 5-hydroxyindole-3-acetic acid (5-HIAA) would be of great value. A mass fragmentographic method would also allow the use of stable isotopes for the investigation of 5-hydroxyindoleamine metabolism[1,2].

Cattabeni *et al.*[3] developed a mass fragmentographic method for the determination of 5-HT using α-methyl-5-HT as an internal standard. However, no extraction procedure was used, which limits the usefulness of the method in the determination of 5-HT in small pieces of brain tissue. The use of deuterated analogues as internal standards is to be preferred as optimal correction for losses during the analytical procedure is obtained.

In this paper, a mass fragmentographic method for the determination of 5-HT and 5-HIAA in the same extract of brain tissue using deuterated analogues as internal standards is described.

MATERIALS AND METHODS

Preparation of brain extracts

Brain tissue was obtained from male Sprague–Dawley rats weighing about 200 g. The animals were injected i.p. with saline (0.9%), pargyline hydrochloride (100 mg/kg), *d,l-p*-chlorophenylalanine methyl ester hydrochloride (PCPA) (500 mg/kg), probenecid (200 mg/kg), tryptophan (300 mg/kg) and reserpine (7 mg/kg). At different time intervals the animals were decapitated and the brain was rapidly removed and dissected on ice. Cerebellum and medulla oblongata were discarded and the remainder of the brain was homogenized in 2× 10 ml of 0.15 *M* formic acid containing ascorbic acid (50 μM). After centrifugation at 26,000 *g* for 40 min, the supernatant was decanted and stored at —75° pending the analysis.

About 1.5 nmole of $\alpha,\alpha',\beta,\beta'$-$D_4$-5-hydroxytryptamine (5-HT-d4)[4] and 2.3 nmole of 5-hydroxyindole-3-(-2-D_2-acetic acid) (5-HIAA-d_2)[4] were added as internal standards to a 3-ml aliquot (equivalent to about 0.2 g of tissue) of the supernatant. After the addition of 1 g of sodium chloride, the samples were extracted with 2× 4 ml of diethyl ether. The remaining aqueous phase and the combined organic phases were processed as described below for the determination of 5-HT and 5-HIAA, respectively.

Determination of 5-HT

To the aqueous phase were added 0.1 g of sodium carbonate, 10 ml of 0.25 *M* borate buffer (pH 10), 4 g of sodium chloride and 15 ml of *n*-butanol and the mixture was shaken and centrifuged. The butanol phase was transferred into a second tube and extracted with 20 ml of *n*-heptane and 1 ml of 0.15 *M* formic acid. After centrifugation, the aqueous phase was removed and lyophilized. Derivatives were prepared by reaction of the residue with 100 μl of pentafluoropropionyl anhydride (PFPA) for 3 h at 60°. After evaporation to dryness, the residue was dissolved in 2 × 100 μl of a 1% solution of PFPA in ethyl acetate and transferred into another tube after removal of the insoluble material. The solvent was evaporated and the residue dissolved in 30 μl of a 1% solution of PFPA in ethyl acetate. The 5-HT derivatives were stable for more than 1 month at —75°.

A 1-μl volume of the sample solution was injected into an LKB 2091 gas chromatograph–mass spectrometer equipped with an accelerating voltage alternator, allowing the simultaneous recording of 4 mass numbers. The column (1.5 m × 2 mm I.D.) was packed with GP 3% SP 2100 on Supelcoport. The trap current was 100 μA, electron energy 70 eV, flow-rate of carrier gas (helium) *ca.* 20 ml/min, column temperature 200°, flash heater temperature 260° and ion source temperature 280°. From the mass spectra of 5-HT and its deuterated analogue the base peaks *m/e* 451 and 454 and the fragments *m/e* 438 and 440 were chosen for the mass fragmentographic analysis of 5-HT (Fig. 1).

Determination of 5-HIAA

The diethyl ether phase from the initial extraction (see above) was evaporated to dryness in a stream of nitrogen. Derivatives were prepared by reaction of the residue with a mixture of pentafluoropropanol and PFPA (1:4) for 15 min at 75° followed by evaporation under vacuum and treatment with PFPA for 5 min at 75° (ref. 5). After evaporation to dryness, the residue was dissolved in 30 μl of a 1% solution of PFPA in ethyl acetate.

A 1-μl volume of the sample solution was injected into an LKB 2091 instrument under conditions according to the method previously described for the determination of 5-HIAA in cerebrospinal fluid[6]. The molecular ions *m/e* 615 and 617 and the fragments *m/e* 438 and 440 were chosen for the mass fragmentographic analysis of 5-HIAA.

The presence of 5-hydroxytryptophol (5-HTOL) was determined by recording the *m/e* 451 peak chosen from the mass spectrum of 5-HTOL (Fig. 2). The possibility that 5-HTOL is present in the brain as a conjugate was tested by incubation of brain extracts with arylsulphatase (29000 U/g, also containing β-glucuronidase, 300000 U/g)[7]. The enzyme was of the type H-I (Sigma, St. Louis, Mo., U.S.A.). For the quantitation of 5-HTOL, 5-HIAA-d_2 (*m/e* 440) served as a standard.

To test the specificity, recovery and experimental error, extracts of three rat brains were pooled and divided into 20 equal portions. To 10 of the samples 587 pmole of 5-HT and 196 pmole of 5-HIAA were added. As a pharmacological test of the specificity of the methods used, 5-HT and 5-HIAA were determined in the brains of rats injected i.p. with different types of drugs that interfere with the metabolism of 5-HT.

Quantitation

Standard solutions were prepared so as to contain the same amounts of the deuterated analogues as mentioned above and different amounts of 5-HT (10–1200 pmole) and 5-HIAA (40–800 pmole). Quantitation was effected by comparing the peak-height ratio for each sample with that of the appropriate standard graph.

RESULTS AND DISCUSSION

As expected from the mass spectrum of 5-HT as the pentafluoropropionyl $(PFP)_3$ derivative[8], our derivative of 5-HT-d_4 showed a molecular ion at *m/e* 618 (Fig. 1). Under the conditions used here, the intensity of the molecular ion is about 3% for the derivatives of 5-HT as well as for the deuterated standard. The deuterated compound demonstrated peak-height ratios less than 1% at *m/e* 614, 618 and 451, 454 and 438 and 440. The base peaks of 5-HT-$(PFP)_3$ and its standard ($M^+ - 163$ and $M^+ - 164$, respectively) and the fragments $M^+ - 176$ and $M^+ - 178$ were chosen for the multiple analysis (Fig. 1). Mass spectra of 5-HIAA and the deuterated analogue 5-HIAA-d_2 as the $(PFP)_3$ derivatives have been published previously[6].

The standard graphs for 5-HT were linear but with different slopes for the two fragment pairs. This result indicates a small difference in the fragmentation pattern between the hydrogen and deuterium species, which may be due to a stronger binding of deuterium than hydrogen to carbon. The multiple ion analysis resulted in approximately the same levels of 5-HT and 5-HIAA at the two mass number pairs used

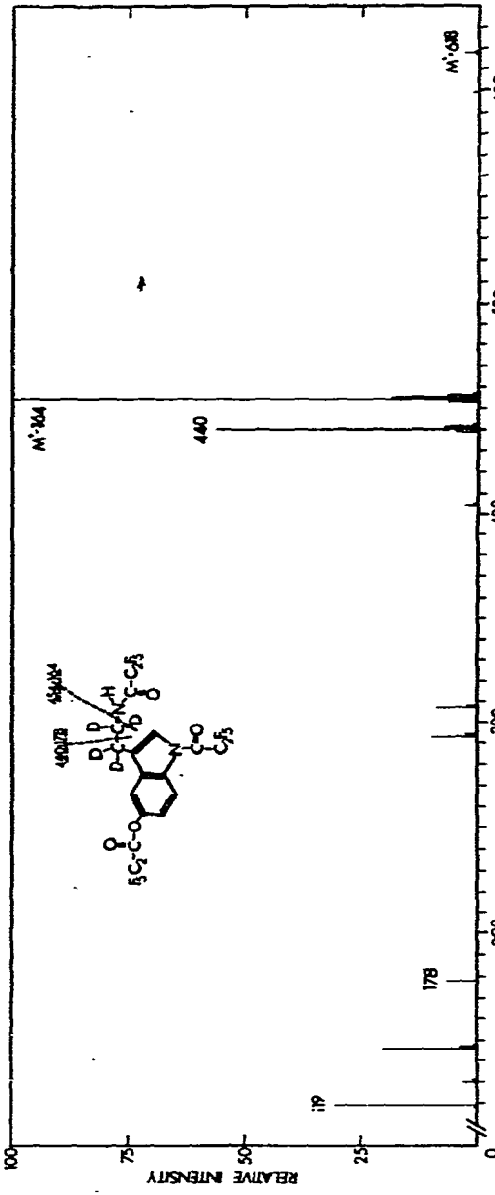

Fig. 1. Mass spectrum of 5-HT-d_4 as the pentafluoropropionyl derivative.

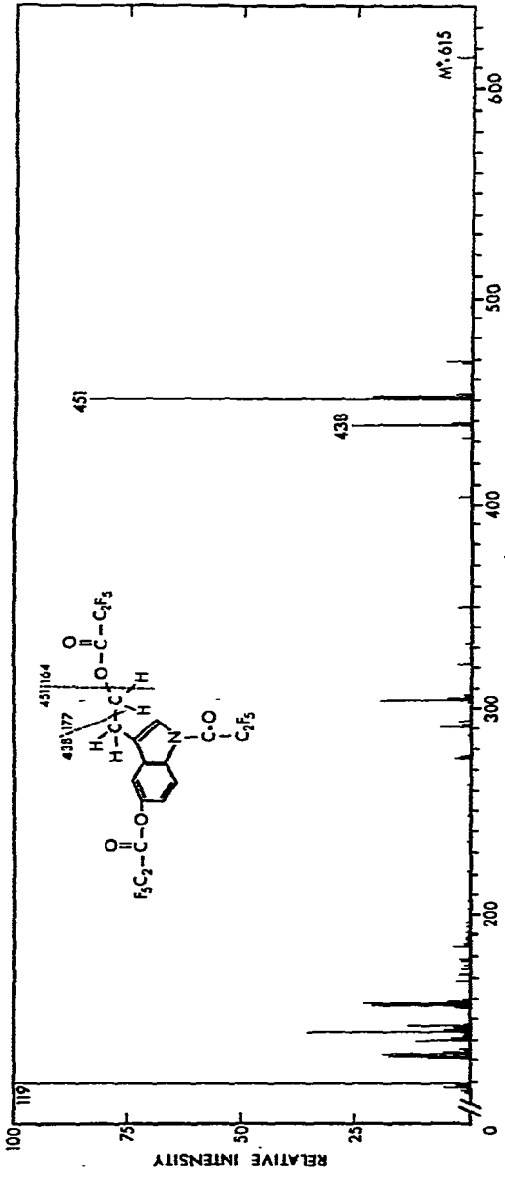

Fig. 2. Mass spectrum of 5-HTOL as the pentafluoropropionyl derivative.

(Table I), demonstrating a high specificity. The experimental error for the mass numbers used in the routine analysis was less than 3% for both 5-HT (*m/e* 451 and 454) and 5-HIAA (*m/e* 438 and 440) (Table I). The recoveries of 5-HT and 5-HIAA added to brain extracts were more than 95% (Table I). The aqueous phase obtained after the extraction of 5-HIAA with diethyl ether could not be used directly for the determination of 5-HT, as a high background was obtained. With the modified extraction procedure described here, mass fragmentograms free from interfering peaks were obtained from the brain extracts (Fig. 3).

TABLE I

MASS FRAGMENTOGRAPHIC DETERMINATION OF 5-HT, 5-HIAA AND 5-HTOL IN POOLED RAT BRAIN EXTRACTS

Treatment	*5-HT*		*5-HIAA*		*5-HTOL, m/e 451, 440*
	m/e 438, 440	*m/e 451, 454*	*m/e 438, 440*	*m/e 615, 617*	
Pool of brain*	522 ± 29	496 ± 15	239 ± 4	248 ± 26	Not detectable
Experimental error (% of mean)	6	3	2	11	—
Pool of brain + 587 pmole of 5-HT and 196 pmole of 5-HIAA per sample*	1078 ± 40	1064 ± 38	438 ± 4	404 ± 29	Not detectable
Recovery (%)	95	97	101	80	—
Pool of brain + 215 pmole of 5-HTOL per sample*	—	—	268 ± 6	278 ± 15	197 ± 13
Recovery (%)	—	—	14	14	92

* Results expressed as picomoles per sample ± S.D.

The mass spectrum of 5-HTOL as the $(PFP)_3$ derivative (Fig. 2) demonstrated that fragments were obtained at the same mass numbers (*m/e* 438 and 615) that were used for the determination of 5-HIAA but also at *m/e* 451. As 5-HIAA-$(PFP)_3$ and 5-HTOL-$(PFP)_3$ could not be separated by the gas chromatograph under the conditions used, the presence of 5-HTOL interferes in the determination of 5-HIAA (Table I). When the presence of 5-HTOL in the brain was examined by recording the *m/e* 451 peak, no peak with a retention time similar to that of 5-HIAA (*m/e* 438) and the standard (*m/e* 440) was obtained, whether or not the brain extracts were subjected to enzymatic hydrolysis. Our results strongly support the conclusions of Duncan and Sourkes[9] that 5-HIAA is the predominant deaminated metabolite of 5-HT in the rat brain, where it occurs principally in the free form[10].

The results of the pharmacological experiments further support the specificity of the 5-HT and 5-HIAA determinations. Thus, the drug-induced changes in the 5-HT levels were the same at both mass number pairs used (Fig. 4, Table II). Moreover,

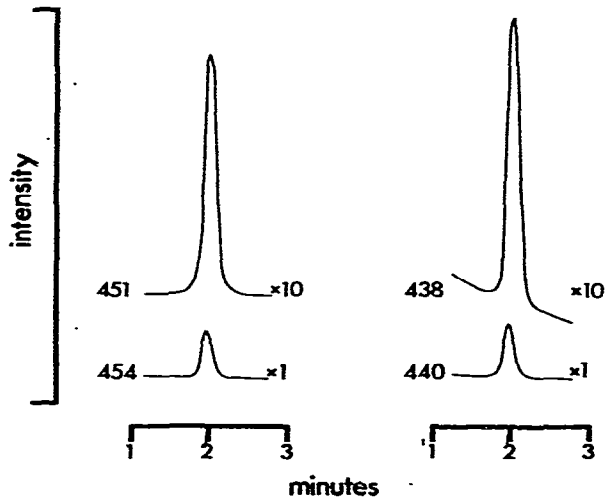

Fig. 3. Mass fragmentograms demonstrating the determination of 5-HT as the pentafluoropropionyl derivative in a brain extract using 5-HT-d_4 as an internal standard.

TABLE II

MASS FRAGMENTOGRAPHIC DETERMINATION OF 5-HT AND 5-HIAA IN THE RAT BRAIN AFTER I.P. INJECTION OF VARIOUS DRUGS

Results are expressed as nanomoles per gram ± S.E. (n = 3–7).

Treatment dose	*Time (h)*	*5-HT, m/e 451, 454*	*5-HT, m/e 438, 440*	*5-HIAA, m/e 438, 440*
NaCl (0.9%)	3	2.95 ± 0.16	2.75 ± 0.16	0.64 ± 0.18
PCPA*		0.99 ± 0.12**	0.93 ± 0.06**	0–0.31***
Probenecid (200 mg/kg)	2	3.57 ± 0.33	3.22 ± 0.25	2.16 ± 0.41§
Pargyline (100 mg/kg)	2	6.06 ± 0.52**	5.82 ± 0.49**	not detectable
Tryptophan (300 mg/kg)	1	4.46 ± 0.31**	4.46 ± 0.36**	2.34 ± 0.58§§
Reserpine (7 mg/kg)	24	0.49 ± 0.11**	0.38 ± 0.14**	1.25 ± 0.15

* PCPA (500 mg/kg) was injected 24 h and 3 h before decapitation.

** $p < 0.001$.

*** In four samples no 5-HIAA was detectable; in the fifth sample 0.31 nmole/g of 5-HIAA was present.

§ $p < 0.01$.

§§ $p < 0.05$.

the drug effects were the same as those reported in previous studies. The inhibitor of 5-HT synthesis, PCPA[11], reduced the levels of both 5-HT and 5-HIAA. Probenecid, which blocks the egress of 5-HIAA from the central nervous system[12,13], increased its content in the brain. Monoamine oxidase degrades brain 5-HT[14] and the blocker of this enzyme increased the 5-HT level concomitantly with a profound decrease in 5-HIAA. Tryptophan treatment resulted in elevated levels of both the amine and the

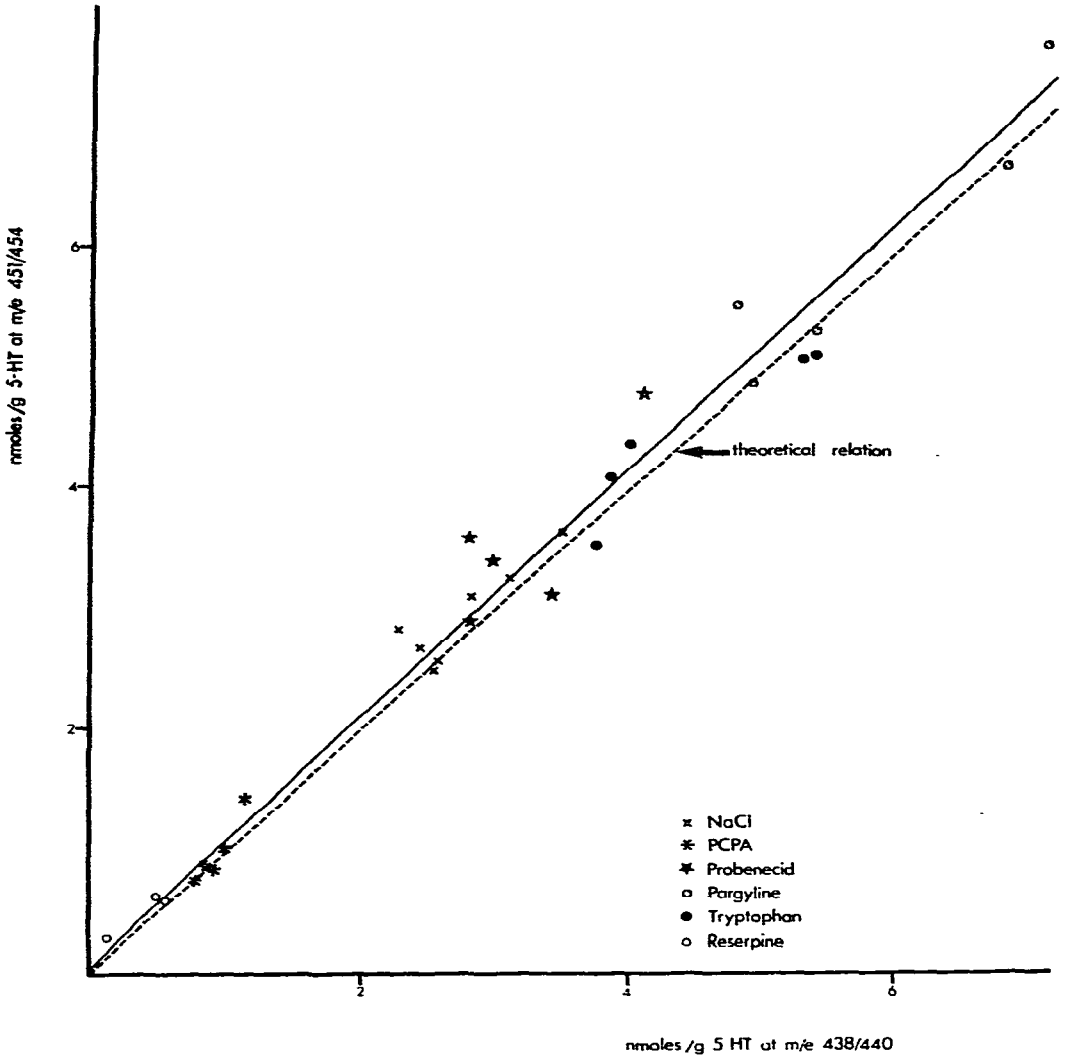

Fig. 4. Mass fragmentographic determination of 5-HT in the brain of drug-treated rats using two different fragment pairs.

acid, supporting the view that the synthesis of 5-HT is partly dependent on substrate availability[15]. Reserpine, a drug known to deplete monoamine stores[16], induced a profound decrease in the 5-HT level and tended to increase the 5-HIAA content.

The mass fragmentographic method described here has a high capacity, allowing the determination of both 5-HT and 5-HIAA in the same brain extract from 100 animals in 3 days. The high specificity and sensitivity of the method allows the determination of the compounds in milligram portions of tissue. The technique also offers the possibility of using precursors that contain stable isotopes such as ^{2}H, ^{18}O and ^{13}C for turnover studies.

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