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MASS FRAGMENTOGRAPHY OF 5-HYDROXYTRYPTOPHOL AND 5-METHOXYTRYPTOPHOL IN HUMAN CEREBROSPINAL FLUID

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SUMMARY

A specific and very sensitive method for the determination of 5-hydroxytryptophol (5-HTOL) and 5-methoxytryptophol (5-MTOL) in extracts from human cerebrospinal fluid (CSF) involving the use of mass fragmentography and pentafluoropropionyl derivatives is described.

5-HTOL and 5-MTOL were determined in human CSF of three patients with leukaemia and from nine patients with neurological disorders. The concentration of free 5-HTOL in CSF was in the range of 0.1–33 ng/ml and that of 5-MTOL was 0.3–13.9 ng/ml.

For the first time the presence of these compounds in human material has been shown. The concentration of these two alcohols in CSF is markedly lower than the concentration of 5-hydroxyindoleacetic acid. These results suggest that human cerebral 5-hydroxytryptamine is preferentially metabolized to 5-hydroxyindoleacetic acid rather than to 5-HTOL and 5-MTOL.

INTRODUCTION

Several workers have reported that disorders of the tryptophan metabolism might play an important role in affective disturbances. Mendels *et al.*[1], Benassi[2], Johannson[3] and Cazzullo *et al.*[4], investigated the tryptophan metabolism of patients suffering from mania, depression, schizophrenia and neurological diseases. Accurate investigations could be made by analyzing brain biopsies[5]. However, the collection of brain biopsies is possible only in extremely severe cases, as was shown for dopamine by Constantinidis *et al.*[6].

Duncan and Sourkes[7] determined the Michaelis constant of the aldehyde reductase (E.C. 1.1.1.2) from pig brain (caudeate nucleus and cerebral cortex) by fluorimetric measurement of the decrease in reduced nicotinamide–adenine dinucleotide phosphate (NADPH) level after incubation with 5-hydroxyindoleacetaldehyde as a substrate. They found that at low aldehyde concentrations, predominantly acidic metabolites are formed, while at higher concentration of the aldehyde, the formation of reduced metabolites (alcohols) is increased.

Sjöquist[8] reported that in human CSF the ratio of 3-methoxy-4-hydroxy-

phenylglycol (MHPG) to 3-methoxy-4-hydroxymandelic acid (VMA) as major catabolites of norepinephrine is about 23 times higher than that in urine, thus indicating that the reduction pathway from norepinephrine to MHPG seems particularly to dominate in the brain. Based on these results, we were also interested in determining the reduced catabolites of 5-hydroxytryptamine (serotonin) in CSF.

Feldstein and Wong[9] found radiographically that after incubation of [^{14}C]-5-hydroxytryptophan in rat liver homogenate, 5-HTOL was produced. This was confirmed by paper chromatography and by the Ehrlich colour reaction.

Iskrić *et al.*[10] developed a fluorimetric method for the determination of 5-HTOL in biological material involving several chromatographic purification steps. However, they did not report the occurrence of this compound in human material. The methylation in catecholamine metabolism, catalyzed by catechol-O-methyltransferase (E.C. 2.1.1.6), leads to inactivation of the amines. 5-Hydroxytryptamine could be inactivated by a similar mechanism. The presence of 5-MTOL might be an indication for this hypothesis.

As only small amounts of 5-HTOL and 5-MTOL were to be expected in human CSF, fluorimetric and gas chromatographic techniques are probably adequate, but they are not sufficiently specific. Mass fragmentography (MF) seems to be the most suitable method for this analysis. Further, this technique makes it possible to carry out loading tests with deuterated precursors of 5-HTOL and 5-MTOL, and the subsequent measurement of these metabolites.

EXPERIMENTAL

Materials

The following chemicals were used: 5-hydroxytryptophol (5-HTOL) (Sigma H-1880; Sigma, St. Louis, Mo., U.S.A.); 5-methoxytryptophol (5-MTOL) (Sigma M-4126); 5-fluoro-α-methyltryptamine hydrochloride (5-FαMTr) (Sigma F-3001); pentafluoropropionic anhydride (PFPA) (Fluka 77292; Fluka, Buchs, Switzerland); trifluoroacetic anhydride (TFAA) (Fluka 91720); bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Regis 270112; Regis, Morton Grove, Ill., U.S.A.); sulphatase (Sigma S-9376); β-glucuronidase (Sigma 105-10); diethyl ether (Fluka 31690, purified according to Vogel[11]); 10% SE-54 liquid phase on 80–100 mesh Chromosorb W AW DMCS (Perkin-Elmer 999; Perkin-Elmer, Überlingen, G.F.R.). All other reagents were of analytical-reagent grade.

Subjects

The patients suffering from leukaemia (cases 1–3) were boys aged 6–10 years. The patients suffering from different neurological diseases (cases 4–12) were three girls and six boys aged 2–18 years.

Collection of cerebrospinal fluid (CSF)

Volumes of 5 ml of CSF were collected in tubes containing 5 mg of ascorbic acid and stored at −20°.

The specimens of patients with neurological disorders were obtained by lumbar puncture during diagnostic pneumoencephalography in general anaesthesia.

The CSF of patients with leukaemia were obtained by lumbar puncture.

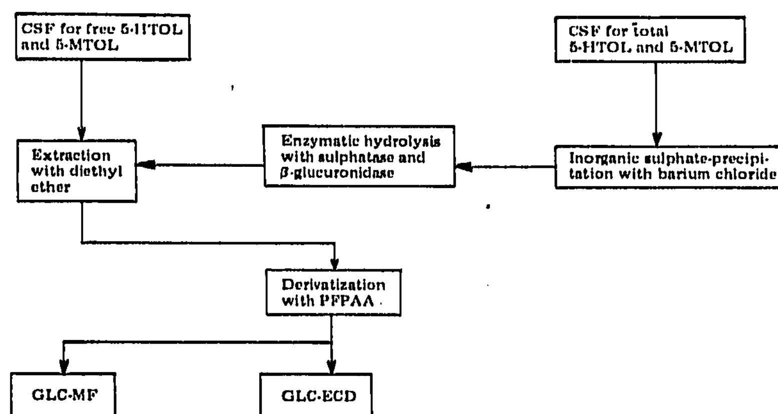

Fig. 1. Schematic diagram of the method for the determination of 5-HTOL and 5-MTOL in CSF.

Treatment of the biological samples

The procedure is shown schematically in Fig. 1.

Extraction of free 5-HTOL and 5-MTOL from CSF

A 1-ml volume CSF was diluted to 5 ml with water. After adjusting the pH to 8.0 with 0.01 *N* sodium hydroxide solution, the solution was extracted with 25 ml of diethyl ether, 20 ml of the ether phase were aspirated off and the CSF was extracted with a further 20 ml of diethyl ether. Then 20 ml of the ether phase were again aspirated off and the combined extracts were concentrated to 2 ml under vacuum at 30°. The concentrated solution was transferred into a 10-ml glass-stoppered tube and the tube was rinsed twice with 1.5 ml of methanol. After the addition of 100 ng of the internal standard (5-FαMTr), the solution was evaporated to dryness under vacuum at 30°. The residue was dried for 10 min in a desiccator over phosphorus pentoxide.

Derivatization

PFPA (100 μl) and acetonitrile (100 μl) were added to the dried sample and the mixture was heated at 60° for 20 min. After cooling to room temperature, the excess of reagents were evaporated under a flow of nitrogen. The residue was dried for 10 min under vacuum over potassium hydroxide–phosphorus pentoxide and dissolved in 100 μl of acetonitrile.

Hydrolysis of conjugated 5-HTOL and 5-MTOL

A 1-ml volume of CSF was diluted to 5 ml with water and the inorganic sulphates were precipitated by adding a saturated solution of barium chloride. The precipitate was centrifuged for 5 min at 500 *g* and the supernatant decanted. After the pH had been adjusted to 6.0 with 0.01 *N* sodium hydroxide solution, 5 mg of sulphatase, 3 mg of β-glucuronidase and one drop of chloroform were added. The sample was incubated for 16 h at 37°.

Gas–liquid chromatography with electron capture detection

Experiments with reference compounds were performed by gas–liquid chromatography (GLC) with an electron capture detector (ECD).

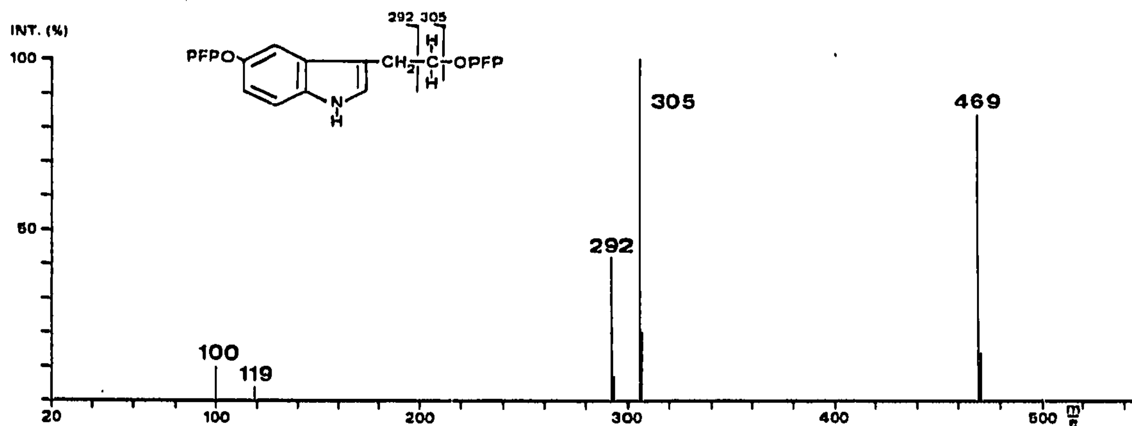

Fig. 2. Electron impact mass spectrum of 5-hydroxytryptophol-PFP at 20 eV.

A Perkin-Elmer Model 900 gas–liquid chromatograph was employed, fitted with a dual flame ionization detector and a ^{63}Ni ECD maintained at 300°.

The glass column (1.80 m × 3 mm I.D.) was packed with 10% SE-54 on Chromosorb W AW DMCS (80–100 mesh). A flow-rate of nitrogen of 30 ml/min was maintained through the column, supplemented with a scavenger flow-rate of 30 ml/min at the column outlet, resulting in a flow-rate of about 60 ml/min through the ECD. The temperatures were as follows: injector block, 250°; column oven, 200°; and manifold, 250°. Volumes of 0.5–1.0 μl from the derivatized solution were injected.

Gas–liquid chromatography–mass spectrometry and mass fragmentography

An LKB (Stockholm, Sweden) Model 9000 GLC–mass spectrometry (MS) instrument was employed. The chromatography column (600 mm × 2 mm I.D.) was filled with 10% SE-54 liquid phase on 80–100 mesh Chromosorb W AW DMCS. The temperatures were as follows: flash heater, 250°; oven, 160°, isotherm; separator, 250°; and ion source, 270°.

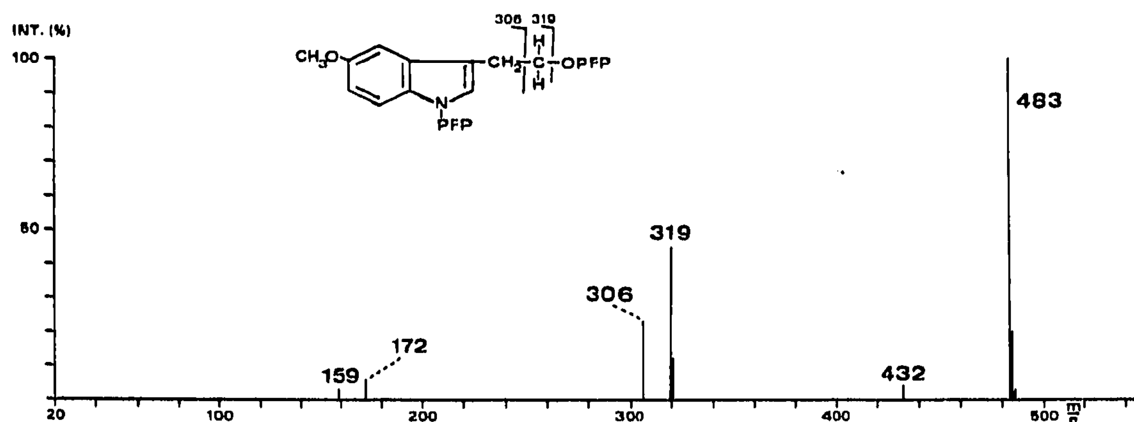

Fig. 3. Electron impact mass spectrum of 5-methoxytryptophol-PFP at 20 eV.

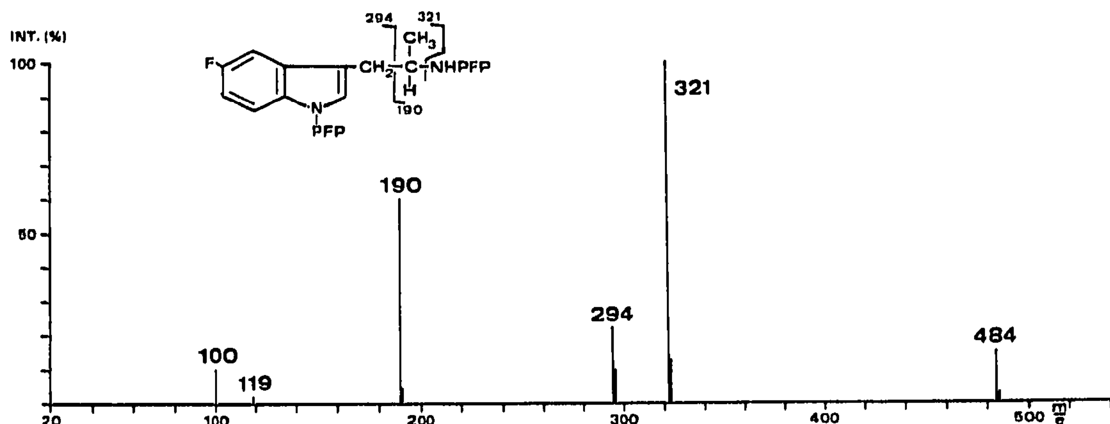

Fig. 4. Electron impact mass spectrum of 5-fluoro-α-methyltryptamine-PFP at 20 eV.

The flow-rate of carrier gas (helium) was 30 ml/min; the accelerating voltage was 3.5 kV and the trap current 60 μA. The split apertures were 0.4 and 0.8 mm.

The MS and MF were carried out at 20 eV. An accelerating voltage alternator device was used for multiple ion monitoring.

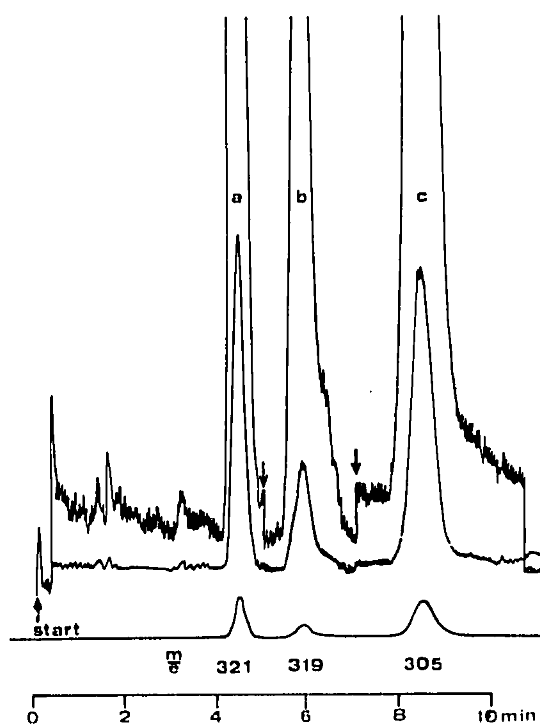

Fig. 5. Mass fragmentogram of free tryptophols in 1 ml of a CSF sample: (a) 5-FαMTr-PFP (*m/e* 321); (b) 5-MTOL-PFP (*m/e* 319); (c) 5-HTOL-PFP (*m/e* 305).

RESULTS

Figs. 2–4 show the mass spectra of 5-HTOL, 5-MTOL and 5-FαMTr as their pentafluoropropionate (PFP) derivatives. The mass spectrum of 5-HTOL-PFP (Fig. 2) is characterized by an intense fragment at *m/e* 305; this fragment results from the cleavage of the bond between the α-carbon and the oxygen with a transfer of hydrogen on to the oxygen atom.

The mass spectrum of 5-MTOL-PFP (Fig. 3) shows an intense fragment at *m/e* 319, which corresponds to the fragment at *m/e* 305 of 5-HTOL-PFP (same cleavage mechanism).

The analysis of 5-HTOL and 5-MTOL was performed by measuring the fragment ions at *m/e* 305, 319 and 321 for the internal standard (an intense fragment of 5-FαMTr-PFP, Fig. 4).

Fig. 5 shows a mass fragmentogram of the internal standard 5-FαMTr-PFP (a), free 5-MTOL-PFP (b) and free 5-HTOL-PFP (c) extracted from 1 ml of CSF. The derivative of the internal standard was measured by the fragment at *m/e* 321, and the derivatives of 5-MTOL and 5-HTOL were measured at *m/e* 305 and 319, respectively. Fig. 6 shows a gas chromatogram with electron capture detection of a 1-ml CSF sample to which 50 ng of 5-HTOL and 50 ng of 5-MTOL were added.

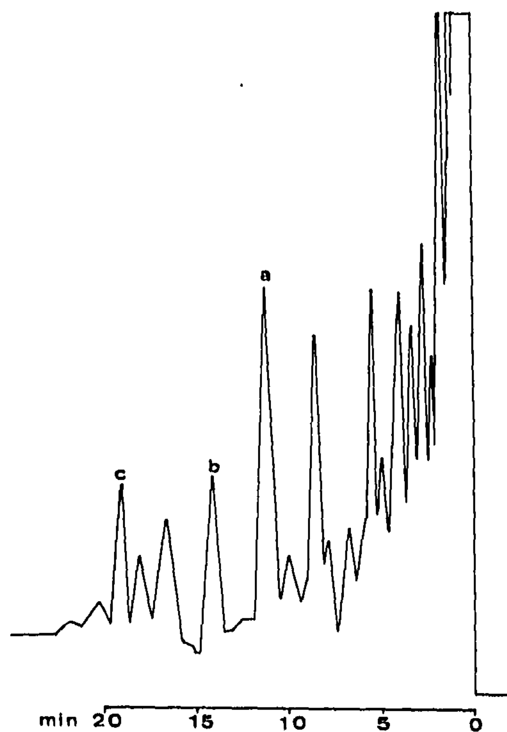

Fig. 6. ECD chromatogram of PFP derivatives of (a) internal standard, (b) 5-MTOL and (c) 5-HTOL Amounts of 50 ng of 5-HTOL and 50 ng of 5-MTOL were added to 1 ml of CSF.

TABLE I

5-HTOL AND 5-MTOL CONCENTRATIONS IN HUMAN CSF

Case No.	*Age*	*Sex*	*Diagnosis*	*Free 5-HTOL per 1 ml CSF (ng)*	*Free 5-MTOL per 1 ml CSF (ng)*
1	6	M	Leukaemia	—*	0.7
2	4	M		10.2	8.4
3	10	M		33.4	7.8
4	12	F	Neurological disorders	—*	13.9
5	2	F		—*	2.8
6	14	M		5.8	6.3
7	4	F		2.8	—*
8	7	M		2.2	5.3
9	6	M		1.7	2.8
10	2	M		0.3	0.3
11	8	F		0.1	0.4
12	18	M		1.1	2.2

* Not determined.

The recovery of free 5-HTOL was 86% and that of free 5-MTOL 89%. The coefficients of variation (n = 7) were 13.0% (5-HTOL) and 13.1% (5-MTOL).

Our results from cerebrospinal fluid are summarized in Table I.

DISCUSSION

In this work, the presence of free 5-HTOL and 5-MTOL in human CSF has been demonstrated for the first time. A method based on MF has been developed for the quantitative determination of 5-HTOL and 5-MTOL and their conjugates.

A gas chromatograph equipped with an ECD was used to analyze reference compounds as their PFP derivatives. However, it is not sufficiently specific for this type of determination in biological material. Investigations using GLC with a nitrogen detector resulted in markedly reduced sensitivity. The use of these methods for biological samples requires additional pre-purification steps[10].

It is assumed that 5-HTOL and 5-MTOL can also occur as conjugates with glucuronic and sulphuric acid. We found that the hydrolysis of the conjugates at pH 1 in the presence of cysteine at 100° for 20 min destroys the tryptophols, in contrast to the suggestion of Korf and Valkenburg-Sikkema[12]. Karoum *et al.*[13] used the sulphatase–β-glucuronidase preparation (suc d'*Helix pomatia*, Industrie Biologique Française) for the hydrolysis of urinary phenolic and indolic metabolites. However, we found that the *Helix pomatia* preparations contain considerable amounts of 5-HTOL and 5-MTOL. Purification of the commercially available enzyme preparations on a Sephadex G-25 column resulted in a loss of activity. On the other hand, β-glucuronidase and sulphatase from Sigma contain only very small amounts of 5-HTOL and 5-MTOL. Nevertheless, with these preparations it is also necessary to include an enzyme blank.

For the quantitative determination of the conjugated 5-HTOL and 5-MTOL, an internal standard is needed that has to pass through the whole analytical process,

TABLE II

INTENSITIES OF MOLECULAR AND SELECTED FRAGMENT IONS OF DIFFERENT DERIVATIVES OF 5-HTOL, 5-MTOL AND 5-FαMTr (INTERNAL STANDARD) AS A PERCENTAGE OF THE INTEGRATED ION CURRENT

Compound	*TFA*		*PFPA*		*TMS*	
	m/e	%	*m/e*	%	*m/e*	%
5-HTOL	465	22.8	469	29.3	393	22.4
	351	45.9	305	34.8	290	46.7
5-MTOL	383	43.5	483	45.0	335	20.9
	269	21.7	319	20.3	232	48.5
5-FαMTr	384	27.5	484	6.9		
	269	25.3	321	38.2		

preferably deuterated 5-HTOL and 5-MTOL. Because these compounds were not available to us, we have not reported any results on conjugates in this paper.

The PFP derivatives were found to be the most suitable for the assay of free 5-MTOL and 5-HTOL by MF. The PFP derivatives allow multiple ion detection at *m/e* 305, 319 and 321 or at *m/e* 469, 483 and 484 (Table II). As shown by the mass spectrum in Fig. 2, 5-HTOL is not acylated at the indolic nitrogen atom under the conditions used for derivatization. The column background of the SE-54 phase is low at the positions of the corresponding fragment ions of the PFP derivatives, which would allow us to use deuterated tryptophols as internal standards without difficulty, and also to measure labelled tryptophols after a loading test with deuterated precursors.

Trifluoroacetyl (TFA) derivatives are less suitable for analysis by GLC and MF. Their ECD response is considerably lower than that of the PFP derivatives. MF detection is possible only at *m/e* 351, 383 and 384, because on our LKB 9000 instrument the recorded masses must not deviate by more than 10% from the full mass.

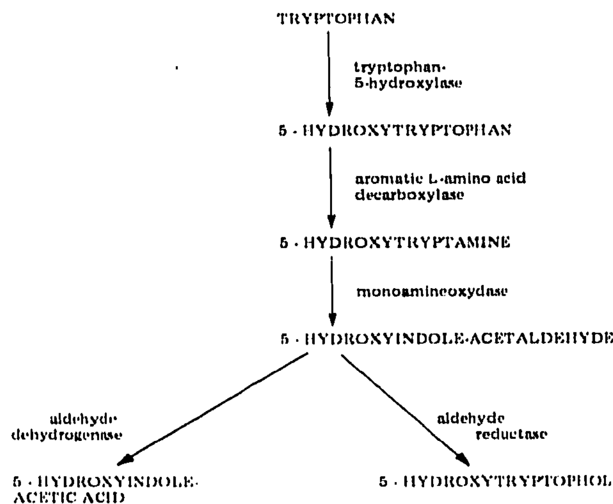

Fig. 7. Metabolic conversion of tryptophan into 5-hydroxyindoleacetic acid and 5-HTOL.

Trimethylsilyl (TMS) derivatives are easily prepared and remain stable for several days. As 5-HTOL-TMS and 5-MTOL-TMS do not have intense fragments within 10% of each other, it was not possible to employ multiple ion detection simultaneously for both compounds.

Tryptophan metabolites might play an important role in affective disorders[1-4]. It has been reported[14] that the reduction pathway from norepinephrine to MHPG dominates, particularly in the brain.

We found the concentration of 5-HTOL, the reduced catabolite of 5-hydroxytryptamine, to be considerably lower in human CSF than that reported for 5-hydroxyindoleacetic acid[15]. These findings suggest that human cerebral 5-hydroxytryptamine is preferentially metabolized to 5-hydroxyindoleacetic acid rather than to 5-HTOL and 5-MTOL, which indicates that the oxydative pathway is preferred.

The catabolism of 5-hydroxytryptamine is shown schematically in Fig. 7. In two cases of leukaemia, 5-HTOL and 5-MTOL were considerably higher than in patients with neurological disorders, as shown in Table I. Of course, three cases are not enough for definite conclusions to be drawn.

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