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Analysis of 5-OH-indoles in human gut biopsy tissues by reversed-phase high-performance liquid chromatography with fluorimetric detection*

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Abstract: A method was devised for the rapid simultaneous determination of major indoles in human gut tissues. Analysis with picomol detection limits was done by HPLC on a C_{18} reversed-phase column with fluorimetric detection at 276/350 nm. This simple method for which there is no necessity of derivatization or purification was validated for routine analysis of small mucosa samples (less than 4 mg fresh weight) obtainable during endoscopy. A comprehensive list of 5-OH-indole compounds in human gut tissue is presented.

Keywords: *HPLC; human gut; indoles; serotonin.*

Introduction

The 5-hydroxyindole derivatives of the essential amino acid tryptophan include some pharmacologically active compounds. Tryptophan (Trp) regulates the 5-HT (serotonin) synthesis in the brain and induces sleep [1–3]. 5-Hydroxytryptophan (5-HTP) and 5-HT are well known as neuroregulators in diverse physiological functions [4].

Several HPLC methods have been reported for the separation of tryptophan and its 5-hydroxyindole metabolites in brain [5–11], cerebrospinal fluid [9], serum [9, 12], and lung tissue [7]. Until now no method has been described for the analysis of gut tissues despite that in mammals most of the body 5-HT is located in the entero-chromaffin cells of the mucosa of the gastrointestinal tract [13] and that the physiological significance of the 5-hydroxyindole pathway in the gut is still not understood [14]. A method is proposed for the simultaneous quantitative assessment of Trp, 5-HTP, 5-HT and 5-HIAA (5-hydroxyindole acetic acid) in human gut tissue; it has been validated for the analysis of small tissue amounts and it is easy to perform under routine conditions. The Trp and 5-hydroxyindole derivatives found in human biopsy specimens obtained under routine gastrointestinal endoscopy have been measured.

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Materials and Methods

Reagents

All chemicals and standards of the highest available purity were obtained from Serva (Heidelberg, FRG). Sodium acetate, citric acid, $HClO_4$ and methanol were purchased from Merck (Darmstadt, FRG).

Apparatus

HPLC was performed with a liquid chromatograph series 10 and a spectrofluorimeter type LS-5 (Perkin–Elmer, Überlingen, FRG). Detection was at 276 nm (excitation) and 350 nm (emission), this being a compromise to obtain maximum sensitivity for all indole compounds. The optimal wavelengths for Trp were excitation at 272 nm and emission at 348 nm and for 5-HTP, 5-HT and 5-HIAA, 276 and 350 nm, respectively. Fluorimeter outputs were monitored on a Perkin–Elmer 561-recorder with a M3B integrator. The analytical column, 250 × 4.6 mm i.d., was used in combination with a short precolumn, 50 × 4.6 mm i.d., both filled with Nucleosil C_{18} 5 μm (Macherey-Nagel, Düren, FRG).

Chromatographic conditions

A citrate–acetate buffer was chosen for its success in earlier studies [15, 16]. After optimization of the different variables the procedure was as follows: mobile phase 0.05 M citric acid/0.05 M sodium acetate/17% methanol, pH 4.3 with no other additive; flow rate 1.0 ml min^{-1}; the mobile phase was prepared with twice distilled water filtered through a Millipore® Q cartridge purification system; the solvent was again filtered just before use with 0.45 μm membranes.

Sample preparation

Standards were prepared on the day of assay by dissolution in 0.01 m $HClO_4$. Human gut biopsies were obtained from major abdominal operations for different gastrointestinal diseases or from endoscopy. Specimens all from patients without gross mucosal lesions were prepared as quickly as possible, cold-stored at 4°C and transported to the laboratory. Immediately thereafter the samples were weighed and, depending on their size, transferred into centrifuge tubes containing 2 ml 0.4 M $HClO_4$ for each 50 mg of tissue. Alternatively the small endoscopic samples (≤4 mg) were placed into small, conical plastic vials.

Homogenization was performed with an Ultraturrax (Janke and Kunkel, Staufen, FRG) or, for small samples, by a rapidly rotating wire loop [17]. After centrifugation at 20,000 **g** for 15 min, 100 μl of the supernatant was injected directly into the chromatographic system.

Results

The most suitable mobile phase variables were selected. After choice of a citrate based buffer in combination with methanol (less toxicity than acetonitrile), the optimal conditions were sought for human gut tissue extracts. The ionic strength of the citric–acetate system could vary between 0.05 and 0.5 M without affecting separation quality on peak shape. The polarity, however, was an important variable. Six different concentrations of the organic modifier methanol (30, 25, 20, 15, 10, 5%) were tested at the same pH value (4.3) in three series. With a methanol concentration of more than

25%, an overlap with less than 10% the peak width at half-height was increased to more than 130% with a tendency to form asymmetric shapes. In an additional series with 7 methanol concentrations (between 25 and 10%), 17% methanol concentration gave maximal difference in retention between 5-HTP and 5-HT without visually affecting peak symmetry and peak width. With this modificiation, run duration was less than 20 min. The detector response, tested over the range 5–350 pmol/100 μl was found to be linear using injected standard solutions. The data were as follows: 5-HTP, $y = 0.2384 + 0.277\ x$ ($N = 9$, $r = 0.9998$); 5-HT, $y = 0.067 + 0.168\ x$ ($N = 9$, $r = 0.9997$); Trp, $y = 0.0421 + 0.158\ x$ ($N = 9$, $r = 0.9997$); 5-HIAA, $y = 0.1202 + 0.051\ x$ ($N = 9$, $r = 0.0001$); where y represents the peak area (arbitrary units from the integrator) and x the concentration of the indole compound (pmol/100 μl).

In some experiments different methods of protein precipitation were evaluated according to their impact on the HPLC performance. The best results were given by 0.4 M $HClO_4$; lower concentrations did not seem to precipitate the protein completely. Other media (trichloracetic acid, methanol or ethanol) gave irregular peaks. The method was adapted to small endoscopic mucosa specimens, usually in the range of 2–4 mg freshweight. A relatively low extraction volume (150 μl) was used with a relatively high injection volume (100 μl) for the chromatographic run. To test the validity of this approach, different amounts of mucosa were extracted in a volume of 150 μl 0.4 M $HClO_4$ and analysed as described. A linear correlation was shown to exist between increasing tissue amounts and fluorimetric values (Fig. 1), and the method was shown to be sensitive enough to analyse endoscopic mucosa samples.

Precision and recovery were tested in 10 consecutively run samples of standards and biopsy extracts (Table 1). Indoles in human gut tissue appeared to be far less stable than standard compounds. Storage at 4°C for 5 h destroyed 13–35%. Freezing to −20°C with thawing after 24 h showed a loss of 20.5–40.2%. This destruction seemed to depend on the kind of tissue. In a different series, samples from the operation theatre containing both mucosa and serosa were obtained. There was a similar degree of instability in both tissue layers during cold-storage at 4°C. However, freezing and thawing affected only the indole content in the mucosa layer.

Extraction of the 5-hydroxyindole compounds from human gut specimens was

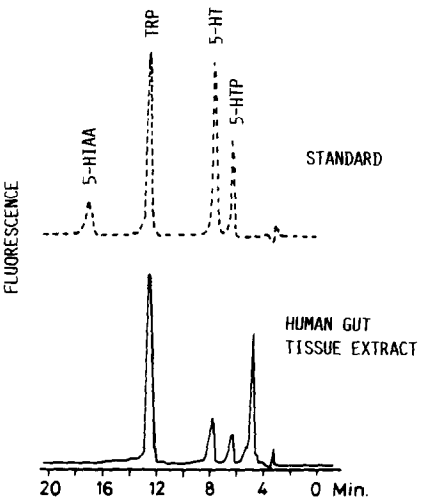

Figure 1
Elution pattern illustrating the separation of indole reference compounds and those in human gut tissue extracts.

Table 1
Data on the technology of the HPLC assay and extraction procedure

	Retention time (min)	Detection limit* (pmol)	RSD† %	RSD‡ %	Recovery§ %
5-HTP	5.3	1.5	2.09	5.46	91
5-HT	6.7	0.9	1.78	9.16	88
Trp	11.4	6.6	5.77	4.87	94
5-HIAA	16.7	6.7	3.78	—	87

*Quantity injected giving a signal-to-noise ratio of 2.
†Relative standard deviation (RSD) for a standard mixture (0.8–1.2 nmol ml^{-1}; $N = 10$).
‡RSD for human mucosa extracts; 10 consecutive samples (*ca* 3 mg/150 μl) from the same mucosa ($N = 10$).
§Recovery findings were based on the peak heights for standards compared with those for pooled mucosa extracts containing added indole compounds (0.8–1.2 nmol ml^{-1}).

therefore performed as soon as possible after preparation and transported to the laboratory. The time interval between endoscopic removal of the mucosa and cooling was less than 1 min in all cases; the transport to the laboratory and the weighing procedure lasted 8.7 min on average (range 7.2–12 min; $N = 10$).

The applicability of the method was tested in five patients admitted for routine endoscopy and without abnormalities of the duodenal mucosa. There was a relatively high concentration of 5-HT and a very low content of 5-HIAA (less than the detection limit of the method) in the human mucosa (Table 2).

Discussion

The lack of data on 5-hydroxyindole compounds in human gut tissues is surprising, since serotonin is more abundant in the gut than in the brain [13]; it is assumed to be implicated in many gastrointestinal disorders [4]. With the method described it is possible to determine simultaneously all metabolites from the serotonin pathway even in the small bioptic specimens which are available during gastrointestinal endoscopy.

There is general agreement in the literature that indole compounds are best separated with HPLC by the use of citrate-based buffers in the eluent [6, 18, 19]. However, Laakso *et al.* [20] recommended phosphate buffer to minimize adsorption problems, but the present results show the significance of this problem is low. The recovery and reproducibility data for the method are good and compare well with other studies [6, 20, 21].

The instability of the 5-hydroxyindole compounds is an aspect which should not be ignored, at least as far as human gut tissue is concerned. In this study, the addition of cysteamine or ascorbic acid to 5-hydroxyindole containing solutions in order to improve their stability [22] was not effective. In fact, the effect appeared to be rather the reverse, because ascorbic acid itself tends to depress the fluorescence signal. Within the tissues 5-hydroxyindole compounds may be released from storage granules [14] and attacked by degrading enzymes (e.g. monoamine oxidase). Presumably the freezing and thawing step promotes such processes, and it can be assumed that gut tissue indoles are particularly susceptible due to the relatively high level of monoamine oxidase activity. Summarizing the observations made in this study, the 5-hydroxyindole compounds in human gut tissues are best extracted under cooled conditions (ice bath) in 0.4 M $HClO_4$ without the addition of antioxidants and without freezing and thawing.

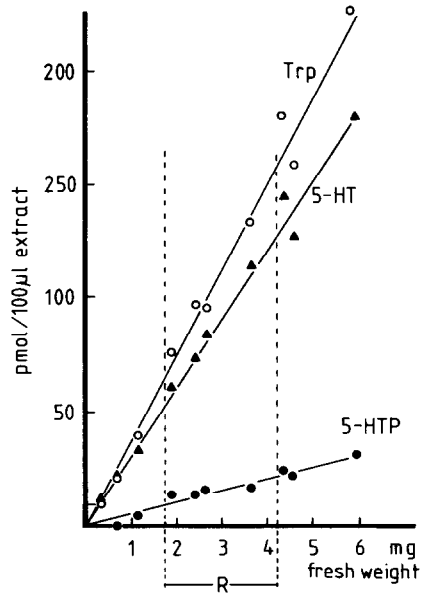

Figure 2
Response curves for increasing amounts of human duodenal mucosa (each extracted in 150 µl of 0.4 M $HClO_4$) and the measured quantities of indole compounds. *R* = range of endoscopic forceps biopsies.

Table 2
Trypotophan metabolite concentrations in human gut tissues

Patient	Diagnosis	Trp	5-HTP	5-HT [pmol mg^{-1}]	5-HIAA
(a) Endoscopic biopsy specimens					
1	Gastric ulcer	40.3	7.6	31.7	—
2	Hiatus hernia with oesophagitis	99.5	32.8	56.7	—
3	Gastric ulcer	36.5	5.0	24.3	2.4
4	Gastric erosions	18.9	12.4	18.0	—
5	No lesion found	41.9	7.2	19.0	—
	Median $\tilde{x}$	40.3	7.6	24.3	—
(b) Specimens from resected ileum during operation					
6	Morbus Crohn				
	Serosa	19.9	6.1	—	—
	Mucosa	36.8	4.6	22.8	—
7	Colon carcinoma				
	Serosa	24.7	4.1	—	—
	Mucosa	58.1	4.0	31.2	—

— = Not found.

HPLC analysis of human mucosa biopsies shows that the spectrum of 5-hydroxyindole compounds in human gut tissue differs completely from that in human brain, both in terms of the absolute amounts present and the relative concentrations. In the brain the amount of 5-HIAA is nearly four times that of 5-HT [8, 22, 24], whereas in the human gut only trace amounts of 5-HIAA could be detected. This may indicate a relatively low turnover (biotransformation) of 5-HT in the human gut, or alternatively a very rapid elimination of 5-HIAA. Perhaps the special endocrine role of 5-HT within the enterochromaffin cells of the gut mucosa [14] is responsible, at least in part, for these biochemical differences.

It should be noted that the kind and amount of food ingested and the clinical condition of the patients involved may well have influenced the 5-hydroxyindole levels in the gut. These variables should be investigated separately and in more detail. Some preliminary observations in different clinical settings showed that there may be a relatively large shift in the Trp/5-HT ratio in the mucosa of patients suffering from gastric carcinoma.

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