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Note

Chromatographic and spectrometric studies on conversion products of 5-hydroxyindole-3-acetic acid

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The instability of 5-hydroxyindole-3-acetic acid (5-HIAA) has been recognized since the 1950s[1–4]. A way of keeping 5-HIAA intact during the analysis procedure as well as in standard solutions and in urine samples was reported in the previous paper[4].

In this communication, studies on conversion products of 5-HIAA are described. The paper presents UV and visible spectra, elution profiles on Sephadex G-10 and screenings of UV-positive constituents in converted states of 5-HIAA by reversed-phase high-performance liquid chromatography (HPLC) with UV-absorbance detection. A preliminary study on 5-hydroxytryptamine (5-HT) conversion is also included.

EXPERIMENTAL

Apparatus

All absorbance measurements were made with a Zeiss spectrophotometer (1-cm quartz cells). For elution on Sephadex G-10 (Pharmacia, Uppsala, Sweden), a peristaltic three-channel pump, P3 (Pharmacia), a fraction collector, FR3 (Stål Produkter, Uppsala, Sweden) and 2 cm × 4 mm I.D. columns were used. Chromatographic conditions were as follows. Total bed volume, V_t = 250 µl; sample volume, 250 µl; flow-rate, 30 µl/min; fraction volume, 225 µl. The absorbance of the individual fractions was monitored at 280 nm. For the HPLC–UV screenings, the previously described[4] equipment and operating conditions were used without modification.

Chemicals and reagents

5-HIAA (Sigma, St. Louis, MO, U.S.A.) stock solutions (1 mg/ml) were prepared in glass-distilled water (pH 5.5) in 0.1 *M* ammonium formate buffer (pH 3.2) and in 0.001 *M* hydrochloric acid (pH 3.2). Elution buffers on G-10 were: 0.1 *M* glycine–hydrochloric acid (pH 4.3) and 0.1 *M* glycine–sodium hydroxide (pH 9.0). The mobile phase solution (pH 2.0) consisted of 110 ml of acetonitrile (p.a. grade from Merck), 890 ml of glass-distilled and degassed water, 0.4 ml of concentrated sulphuric acid and 100 mg of sodium lauryl sulphate.

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Procedure

Conversion states of 5-HIAA were obtained by storing stock solutions at low pH in the experimental conditions described in the table text and figure legends. In order to study the influence of pH on the colour of the obtained solution as well as on the elution behaviour on Sephadex G-10, a few millilitres of a stock solution in distilled water was exposed to bright daylight. For G-10 chromatography, the pink solution obtained was diluted (after being centrifuged) with an equal volume of the buffer of pH 4.3 and 9.0, respectively. To check the pH interval of the colour shift, pH-metric titrations were made on undiluted solution by addition of 0.1 ml portions of 0.002 *M* sodium hydroxide solution. HPLC-UV screenings of stock solutions stored under different conditions were performed as previously[4] on the reversed-phase column, Partisil-10 ODS, 25 cm × 4.6 mm I.D. (Whatman, Clifton, NY, U.S.A.).

RESULTS AND DISCUSSION

It was previously found[4] that the content of intact 5-HIAA in solutions stored under room conditions decreases relatively quickly in bright daylight at pH above 5 as well as in the presence of chloride ions. Concentrated 5-HIAA solutions exposed

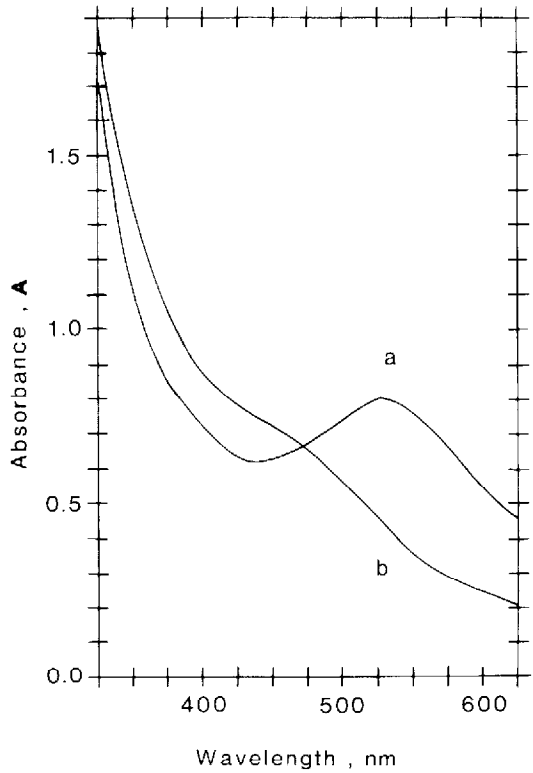

Fig. 1. Visible spectrum of the converted form of 5-HIAA (a) at pH 4.3 and (b) at pH 9.0 (see *Procedure*). (Intact 5-HIAA solutions: colourless.)

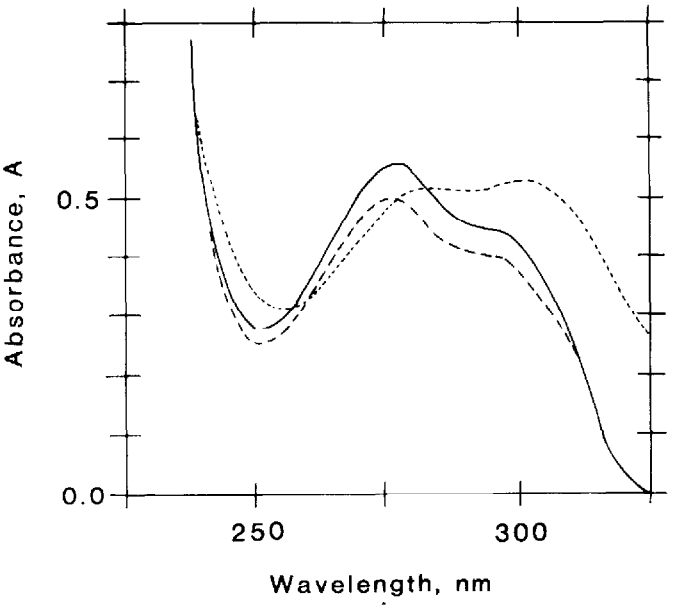

Fig. 2. UV spectra of intact and converted 5-HIAA stock solutions. (———) Freshly prepared stock solution in 0.1 *M* ammonium formate buffer, pH 3.2; stock solutions in 0.001 *M* hydrochloric acid stored at −20°C in the dark (– – – –) for 6 and (.....) 35 weeks. Spectra monitored on 50 times diluted solutions.

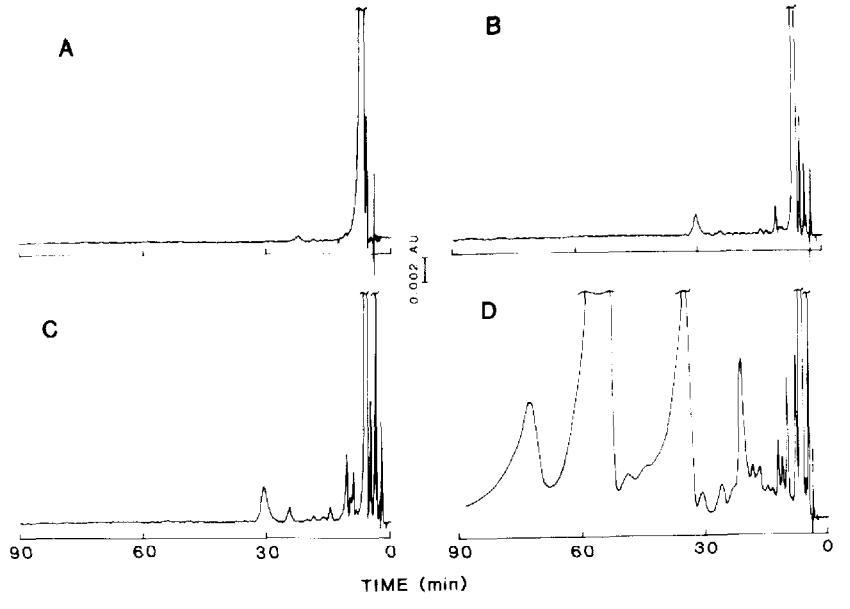

Fig. 3. HPLC UV screening of intact and converted 5-HIAA stock solutions. (A–C) In 0.1 *M* ammonium formate buffer, pH 3.2: (A) freshly prepared; (B and C) stored at room temperature in ambient daylight for 1 week, (B) in tinfoil-wrapped and (C) in transparent test-tubes. (D) Solution in 0.001 *M* hydrochloric acid stored at −20°C in the dark for 35 weeks. (HPLC–UV conditions as in ref. 4.)

to sunshine become coloured (A_{max} at 530–540 nm) already after 2–3 days storage, whereas stock solutions in ammonium formate buffer, pH 3, can be kept intact for several months if stored at −20°C in the dark. The present study shows that 5-HIAA stock solutions in distilled water become pink within 2–3 days storage even in the dark when exposed continuously to a high temperature (40–48°C). All the investigated 5-HIAA solutions, which became pink, changed pH from 3.2, respectively 5.5, to 4.1 ± 0.2. Furthermore, the converted product behaved as an indicator, shifting its colour (pink ⇌ yellow) according to the degree of acidity/alkalinity, the colour change encompassing a pH interval of 7.3–7.9. About 4.5 ml of 0.002 *M* sodium hydroxide was needed to titrate 1 ml of such a pink (A_{530} = 1.100) solution (pH 3.9) to a yellow one (pH 7.9). The converted form of 5-HIAA at low pH is characterized by an absorption maximum at 530 nm in its visible spectrum (Fig. 1, curve a). It may be added that 5-HIAA solutions at low pH kept in the dark at 48°C continuously for 2 weeks, as well as those exposed to sunshine at temperatures temporarily rising to 50°C, showed a further absorption maximum at 340 nm. The visible spectrum of the converted form at high pH does not have a maximum (Fig. 1, curve b). Fig. 2 illustrates changes in the UV spectrum caused by the conversion. HPLC UV elution patterns of converted states are shown in Fig. 3. As seen in Fig. 3D, the conversion product (A_{530} = 2.000) could be resolved into 21 components on the ODS column at pH 2 with the chosen ion-pairing reversed-phase conditions (the

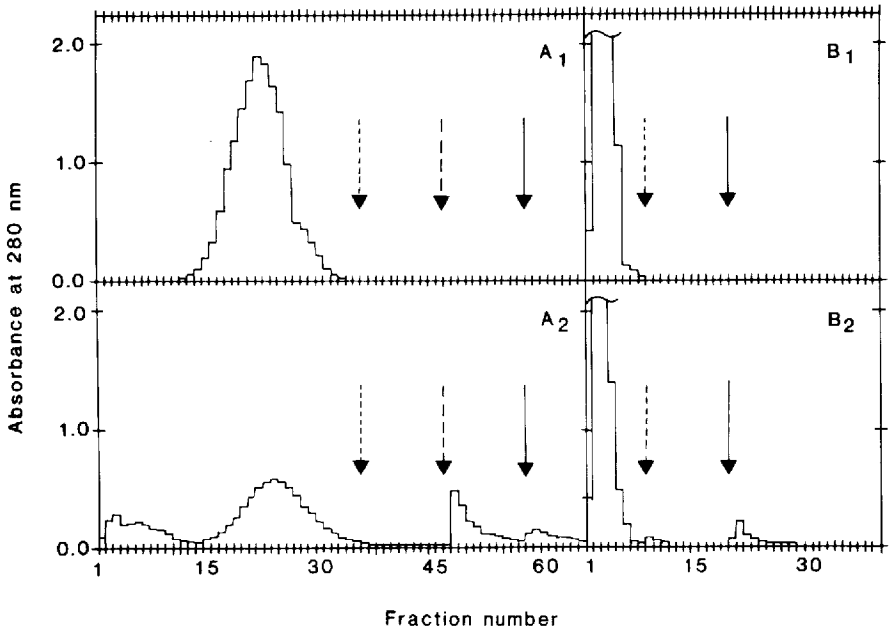

Fig. 4. Sephadex G-10 chromatograms of intact (A_1 and B_1) and converted (A_2 and B_2) 5-HIAA stock solutions. Freshly prepared stock solution in distilled water diluted with an equal volume of buffer (A_1) at pH 4.3 and (B_1) at pH 9.0 and eluted with the respective buffer. Converted 5-HIAA (A_{530} = 1.600) solution (for sample preparation, see *Procedure*) eluted with the buffer (A_2) at pH 4.3 and (B_2) at pH 9.0. Arrows indicate elution with (.....) buffer containing 2 *M* sodium chloride; (---------) buffer, pH 9.0; and (——) 50% ethylene glycol in the buffer, pH 9.0. (Chromatographic conditions as in *Apparatus*.)

absorbance of the effluent was monitored at 280 nm only; no absorbance was detectable at 530 nm). On the other hand, intact 5-HIAA elutes as a single peak at retention time t_R = 6.5 min, except that concentrated solutions (1 mg/ml) show also a minor impurity peak at 5.8 min (diagram A). The major part of the components of the converted product was extremely retarded, probably owing to hydrophobic bonding as well as ion-pairing partitioning of the constituents.

A comparison of the retention behaviours of some phenolic acids and their hydroxyl derivatives on G-10 at low pH suggests that 5-HIAA is one of the most strongly adsorbed of the aromatic compounds investigated[5]. In order to check the adsorption behaviour of the conversion product, G-10 chromatography was performed on a mini-column (sample volume = 1 V_t) at pH 4.3 and 9.0, respectively, and the elution behaviour was compared with that of intact 5-HIAA under corresponding experimental conditions (Fig. 4). As is seen in the chromatogram A_2, at pH 4.3 the pink product was resolved into two separate peaks with V_e/V_t values (V_e = elution volume) of 2.7–3.5 and 21.6–22.4, respectively. Since only the upper half of the column was pink after elution with 32 bed volumes of buffer and none of the collected 36 fractions showed any absorbance at 530 nm, further elution was carried out with buffer of increased ionic strength, at higher pH and lower polarity. Thus, increasing the ionic strength showed no effect, whereas raising the pH and lowering the polarity of the eluent led to recovery of more of the adsorbed substance, indicating the presence of hydrophobic interactions. The elution position of intact 5-HIAA at pH 4.3 is given in chromatogram A_1 (V_e/V_t = 19.8–20.6). On the other hand, at pH 9.0 both intact 5-HIAA (B_1) and its yellow conversion product (B_2) eluted between the fractions 1–8 and appeared as single peaks only (V_e/V_t = 1.8–

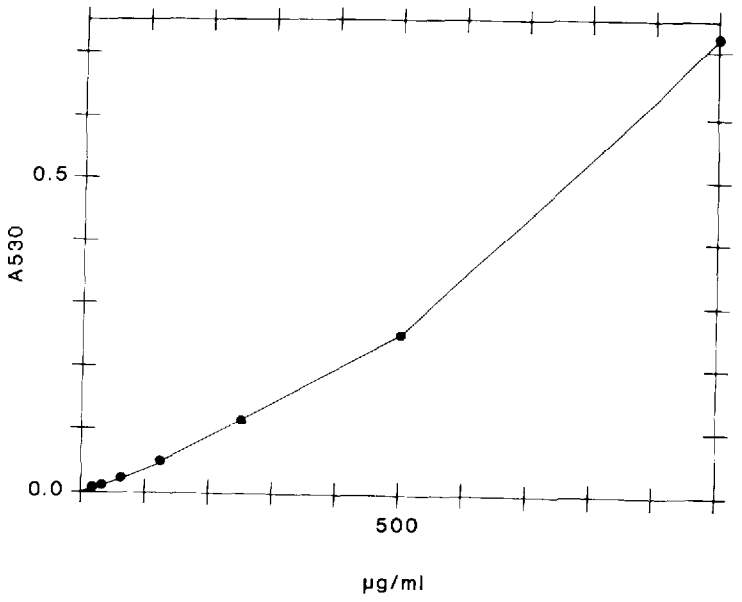

Fig. 5. 5-HIAA concentration influence on the colour intensity of converted states of 5-HIAA at low pH. Stock solution and serial dilutions in 0.1 *M* ammonium formate buffer, pH 3.2, stored exposed to bright daylight for 2 weeks.

TABLE I

COLOUR INTENSITY OF CONVERTED 5-HIAA SOLUTIONS

A_{530} of 1 mg/ml stock solutions in distilled water stored as described below in (a) transparent and (b) tinfoil-wrapped glass tubes.

Storage time	*In room in ambient daylight, at 20°C**		*At window, exposed to sunshine*				*In thermostat*	
	a	*b*	*40°C**		*50°C**		*40°C***	*48°C***
			a	*b*	*a*	*b*	*b*	*b*
1 week	–	–	0.505 0.000***	0.034	0.605	0.080	0.247	0.520
2 weeks	0.070	0.085	1.340§	0.155	1.390§	0.215	–	0.860§

* Maximum temperature in daytime.
** Constant temperature.
*** In nitrogen atmosphere, respectively in the presence of 4 mg/ml sodium bisulphite.
§ Dark sediment was formed and removed.

2.6). However, the converted product was not completely recovered; still more substance was desorbed by increasing the ionic strength and lowering the polarity of the eluent. (Fractions 2–4 in B_2 and 48–49 in A_2 were yellow.)

The data presented in Table I (see also Fig. 3B and C) make it clear that, besides the expected light dependency, the temperature factor is important for the conversion process. As Fig. 5 illustrates, the conversion is also influenced by the 5-HIAA concentration. Furthermore, the colouration starts on the surface of the so-

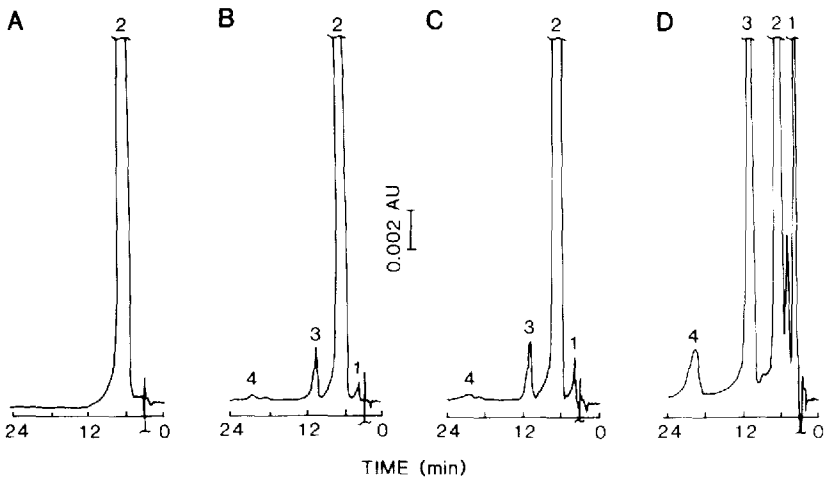

Fig. 6. HPLC–UV screenings of 5-HT stock solutions (0.5 mg free base/ml) in distilled water. (A) Freshly prepared solution, (B–D) solutions stored at *ca.* 20°C; (B) in the dark for 1 week, (C) in ambient daylight for 1 week and (D) in ambient daylight for 8 weeks. (Equipment and operation conditions as in ref. 5; mobile phase, 300 ml acetonitrile–700 ml glass-distilled and degassed water–0.4 ml concentrated sulphuric acid–100 mg sodium lauryl sulphate.)

lution (ocular observations) and does not occur in the presence of sodium bisulphite or in oxygen-free solutions (Table I), which indicates an oxygen dependency of the conversion phenomenon.

Publications on the photochemical behaviour of indole and imidazole derivatives report the reaction pathways and products of some amino acids, mainly tryptophan and histidine[6–10]. The present study of 5-HIAA conversion behaviour may be of use for further investigations, such as the characterization of conversion constituents and exploring the phenomenon.

A comparative study of the conversion of the closely related indolic compound 5-HT (creatinine sulphate complex from Sigma) resulted in the following findings: no light dependency was observed; concentrated solutions (0.5 mg free base/ml) at pH 7–8 showed an increasing turbidity after 1–2 weeks storage under room conditions, contrasting with slow conversion at pH 4; concentrated as well as dilute solutions of 5-HT in converted states become yellow-brown at both low and high pH. A typical HPLC–UV pattern of converted 5-HT solutions is shown in Fig. 6D.

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