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Note

Determination of urinary tryptophan, 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in neonatal hyperbilirubinaemic infants using reversed-phase high-performance liquid chromatography with fluorescence detection

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5-Hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) are related as neuroregulators to physiological function (sleep regulation and sexual behaviour) [1, 2]. Tryptophan regulates 5-HT synthesis in the brain through a rate-limiting enzyme, tryptophan hydroxylase [3—7]. Tryptophan is known to be photosensitive [8—13]. We demonstrated that tryptophan promotes the photodecomposition of bilirubin in the presence of riboflavin [14]. Rubaltelli et al. [15] and Antoni et al. [16] also demonstrated that the urinary excretion of tryptophan metabolites of the kynurenic pathway decreased in hyperbilirubinaemic infants treated with phototherapy.

Recently several methods have been reported for the simultaneous separation of tryptophan, 5-HT and 5-HIAA in brain [17—24], cerebrospinal fluid [23], serum [19, 23, 25, 26] and lung tissue [20]. Some reports have described an analysis for tryptophan metabolites in urine [27—33]. We propose a method for the simultaneous quantitative assessment of tryptophan, 5-HT and 5-HIAA in urine. We measured urinary tryptophan and its metabolites in hyperbilirubinaemic infants treated with phototherapy.

EXPERIMENTAL

Reagents

The reagents used were all of the highest purity. L-Tryptophan and 5-hydroxytryptamine were purchased from Wako (Tokyo, Japan), 5-hydroxyindoleacetic acid was from Aldrich and 5-hydroxytryptophan (5-HTRP)

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from Sigma (St. Louis, MO, U.S.A.). Disodium hydrogen phosphate, potassium dihydrogen phosphate, acetic acid, sodium acetate, sodium sulphate and anhydrous methyl alcohol were obtained from Wako. A creatinine kit was purchased from Daiichi Chemical Co. (Tokyo, Japan).

Apparatus

Chromatography was performed with a Hitachi 635 S pump, and fluorescence detection was accomplished using a Hitachi 650-60 fluorescence spectrophotometer with an 18-μl flow cell (excitation 285 nm, emission 340 nm). Monitor outputs were recorded on a Hitachi 050 recorder. The column was a stainless-steel Unicil C_{18} (25 cm × 4 mm I.D., 10 μm particle size) obtained from Gaskuro (Tokyo, Japan). Sample injections were made with a 100-μl Hamilton syringe.

Chromatographic conditions

Stock solution consisted of 4.8 g of acetic acid, 1.64 g of sodium acetate and 28 g of sodium sulphate in 1000 ml of distilled water. The low concentration eluent (solvent 1) was made by diluting the stock solution with distilled water (1:1, v/v). The high concentration eluent (solvent 2) was a mixture of stock solution—water—anhydrous methyl alcohol (4:3:1, v/v). The column was equilibrated to solvent 1 for at least 10 min before injection. Solvent 1 flowed for just 10 min after sample injection and was followed by solvent 2 for 20 min. The flow-rate was 1.0 ml/min.

The column was stored in 50% methyl alcohol when not in use.

Sample preparation

Standard solutions of each compound were prepared by dissolving the respective compounds in 0.2 *M* phosphate buffer pH 5.6. Urine samples from fourteen hyperbilirubinaemic infants treated with phototherapy were collected before treatment, after 48 h of phototherapy and 24 h after the treatment was brought to an end. The urine was stored at −18° C. Samples were centrifuged at 300 *g* for 10 min and filtered through a Millipore membrane, pore size 0.45 μm (Millipore, Bedford, MA, U.S.A.); 20 μl of each sample were injected on the column. These procedures were performed in dimmed light. The concentration of each compound in urine was determined by its peak height and expressed in μmol/g of creatinine.

Photoirradiation in vitro

Urine was put in a petri dish with 10 μg/ml riboflavin, and was illuminated under a blue white light (intensity 3 mW/cm^2/sec, FL 20BW-NU, National Co., Osaka, Japan), which cuts off ultraviolet rays.

Identification of peaks

Initial identification of the peaks of interest was based on retention time and co-chromatography with the reference compounds. The peaks of samples were compared with stopped-flow spectra of the reference compounds.

RESULTS

The separation of a test mixture of tryptophan, 5-HT and 5-HIAA by two-step gradient with low and high eluents is shown in Fig. 1a. Analysis of these compounds was complete about 30 min after injection.

The reproducibilities of the peak heights were 5.2% (tryptophan), 5.1% (5-HT) and 3.4% (5-HIAA) as shown in Table I. The system was routine tested with a standard mixture before analysis of the samples. Retention times of all compounds showed daily variations of up to 1.0%.

Detection by fluorescence was found to be sensitive at excitation 285 nm and emission 340 nm for these substances. Standard curves of peak height versus amount injected were linear in the range of at least 15—300 pmol for tryptophan, 3—75 pmol for 5-HT and 25—750 pmol for 5-HIAA.

Chromatograms of urine of the newborn infants are shown in Fig. 1b. Tryptophan, 5-HT and 5-HIAA were all detected in basal urine. The peak of 5-HTRP overlapped an unknown peak. The identification of the compounds was performed by spiking the urine samples with the standards. The stopped-flow spectra of each peak corresponded to those of the standards as shown in Fig. 2.

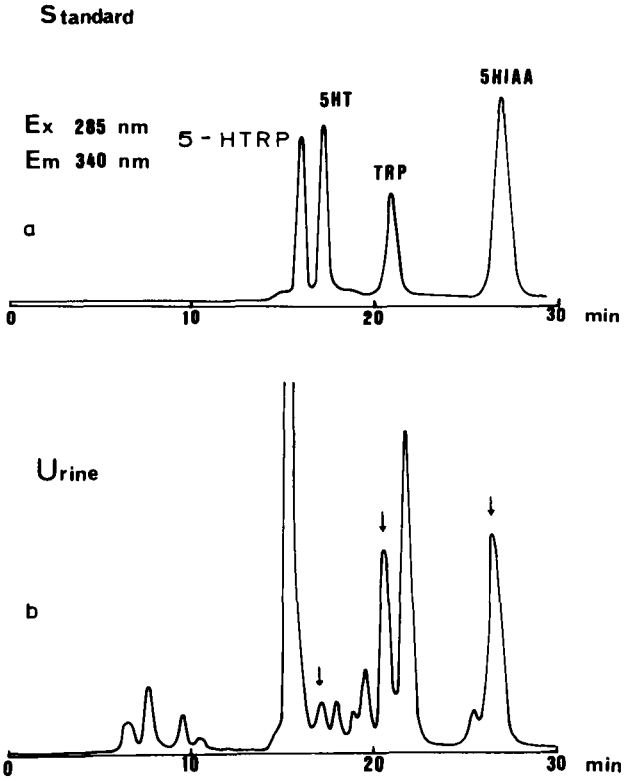

Fig. 1. (a) Chromatogram illustrating the separation of reference compounds detected by native fluorescence with an excitation wavelength of 285 nm and emission cut-off filter of 340 nm. The amounts of 5-HTRP, 5-HT, tryptophan (TRP) and 5-HIAA are 50 pmol, 50 pmol, 200 pmol and 500 pmol, respectively. (b) Separation of the urine sample.

TABLE I

REPRODUCIBILITY

	Peak height (mm)			Retention time (min)		
	Mean	S.D.	C.V. (%)	Mean	S.D.	C.V. (%)
5-HT	76.0	3.9	5.1	8.6	0.1	0.8
TRP*	60.2	3.1	5.2	10.3	0.1	0.8
5-HIAA	146.5	5.6	3.8	13.2	0.1	1.0

*TRP = tryptophan.

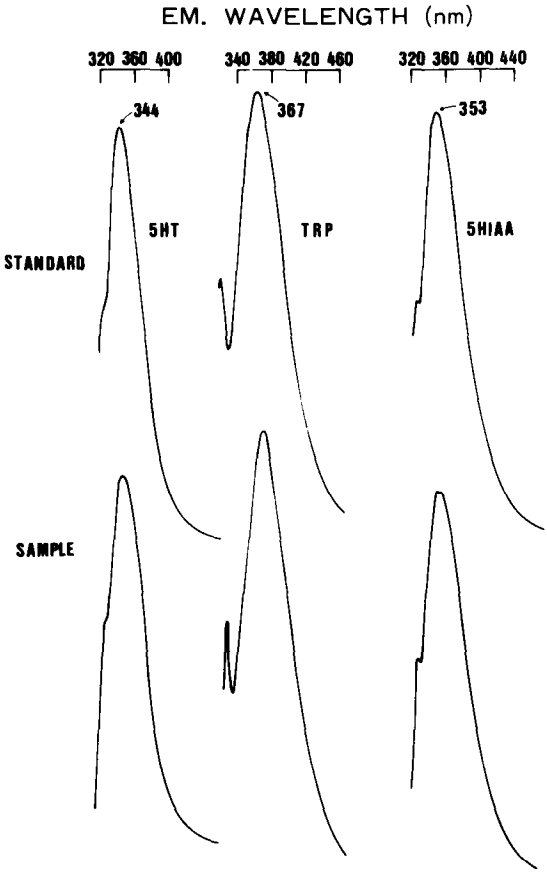

Fig. 2. Stopped-flow spectra of the reference compounds and the urine sample.

The concentrations of urinary tryptophan, 5-HT and 5-HIAA before phototherapy were 43.5, 1.4 and 49.4 μmol/g of creatinine, respectively. In in vitro study, the amounts of tryptophan, 5-HT and 5-HIAA in the urine decreased markedly by photoirradiation (Fig. 3). However, the urinary excretion of these compounds was not influenced by phototherapy in the infants with hyperbilirubinaemia (Table II).

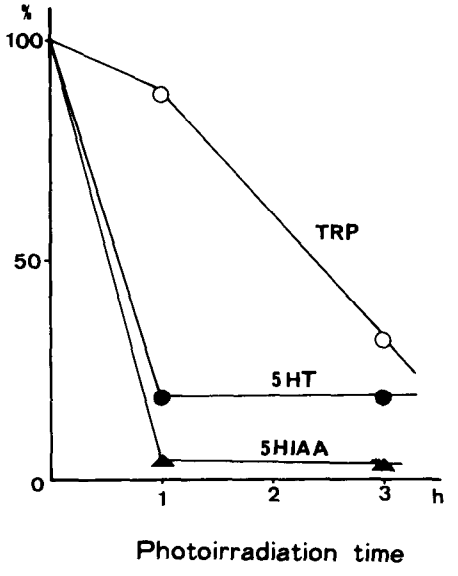

Fig. 3. Photodecomposition of tryptophan metabolites in vitro.

TABLE II

EFFECTS OF PHOTOTHERAPY ON TRYPTOPHAN METABOLITES IN URINE

Results are given as μmol/g of creatinine (mean ± S.D.).

	Before therapy	After 48 h of therapy	24 h after end of therapy
TRP	43.5 ± 8.1	46.1 ± 10.4	50.9 ± 9.6
5-HT	1.4 ± 0.2	1.4 ± 0.3	1.5 ± 0.3
5-HIAA	49.4 ± 4.6	50.9 ± 7.9	47.4 ± 9.4

DISCUSSION

Several methods have been employed for the separation of urinary tryptophan and its metabolites: Denkla's method, ion-exchange [29, 32, 33], and straight-phase [28] and reversed-phase high-performance liquid chromatography [27, 30, 31]. The method described in the present report is suitable for analysis of very small samples with minimum quantities of tryptophan and its metabolites. The sensitivity of the determination was found to be suitable for the analysis of urinary tryptophan and its metabolites in neonatal urine without tryptophan loading. This method guarantees a quantitative determination of tryptophan and its metabolites via 5-HT.

It has been shown that, in jaundiced infants treated with phototherapy, the excretion of tryptophan metabolites of the kynurenine pathway (kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid) was lower in comparison with untreated infants [15, 16]. When the urine of jaundiced newborn infants was exposed to light in vitro, tryptophan and its metabolites via 5-HT decreased significantly. However, the excretory pattern of tryptophan metabolites via 5-HT in jaundiced newborn infants after phototherapy did not differ from that before phototherapy.

There is no evidence that the photoirradiation directly induces the alterations in vivo. We stress the importance of defining the dose–effect relationship in phototherapy to avoid overexposure of newborn infants.

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