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Note

Determination of plasma 5-hydroxytryptophan, 5-hydroxytryptamine, 5-hydroxyindoleacetic acid, tryptophan and melatonin by high-performance liquid chromatography with electrochemical detection

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Following reports in recent years on abnormal platelet functions in preeclampsia [1], increasing attention has been focused on the pathophysiological role of serotonin (5-hydroxytryptamine; 5-HT) [2], and several high-performance liquid chromatographic (HPLC) methods for the determination of this substance have since been developed [3–7]. However, the determination of 5-HT in blood is restricted to the 5-HT contained within platelets, which is pharmacologically inactive. Plasma 5-HT, which is active, is more difficult to determine because it is present in very small amounts and has therefore been the subject of few systematic studies.

Melatonin is synthesized from 5-HT in the pineal gland. The large body of experimental data now available suggests that a pineal–gonadal feedback mechanism may be involved in the control of reproductive function [8,9]. However, no HPLC system sensitive enough to detect plasma melatonin has yet been reported.

This paper describes the methods employed in our laboratory for the determination of tryptophan (TRP) and its metabolites in maternal and unbilical cord plasma and of plasma melatonin by means of reversed-phase HPLC with electrochemical detection.

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EXPERIMENTAL

Chemicals

5-Hydroxytryptophan (5-HTP), 5-hydroxyindoleacetic acid (5-HIAA) and melatonin were obtained from Aldrich (Milwaukee, WI, U.S.A.) and L-TRP, 4-hydroxyphenylacetic acid (HPA) and 5-HT creatinine sulphate from Wako (Osaka, Japan). Stock solutions of the reference and internal standard (HPA) were prepared in 0.1 *M* perchloric acid at a concentration of 100 μg/ml and stored at −80°C.

Apparatus

The HPLC system was obtained from IRICA Instrument (Kyoto, Japan) and consisted of a Chromatic p-321 pump, a Model E-308 amperometric detector with a glassy carbon electrode and a stainless-steel column (250 mm×4 mm I.D.) packed with 10-μm IRICA RP-18. The mobile phase used for the separation of the compounds was a mixture of 0.1 *M* sodium acetate, 0.1 *M* citric acid (pH 4.1) and 0.03 m*M* Na_2EDTA, and 5–30% acetonitrile or 10–37% methanol. All separations were performed at a flow-rate of 0.5 ml/min.

Preparation of samples

Samples for the determination of melatonin were obtained from women (22–42 years old) with normal menstrual cycles. Lights were on from 6 a.m. to 9 p.m. Blood was drawn from the antecubital vein between 7 and 8 a.m., between 5 and 6 p.m., between 10 and 11 p.m. and between midnight and 1 a.m. Serum samples were stored at −80°C until analysed. Volumes of 2 ml of serum were pipetted into 20-ml glass-stoppered tubes and 12 ml of chloroform and 0.2 ml of 0.1 *M* sodium hydroxide solution were added. The mixture was shaken for 10 min and centrifuged, and 11 ml of the chloroform layer were taken and washed twice with 2 ml of distilled water. After further centrifugation, 10 ml of the chloroform layer were collected and dried under vacuum. The residue was dissolved in 0.5 ml of chloroform three times and dried under a stream of nitrogen at room temperature. The residue was dissolved in 50 μl of the eluent and samples of 40 μl were used for the analysis.

For the determination of 5-HTP, 5-HT, 5-HIAA and TRP, samples of 4 ml of maternal venous blood and umbilical arterial and venous blood were obtained in heparinized syringes and transferred into siliconized tubes containing 0.1 ml of 10% Na_2EDTA. Platelet-free plasma was obtained by further centrifugation (2500 *g*) for 15 min at 4°C. An aliquot of 200 μl of the supernatant was transferred into a test-tube containing 100 ng/10 μl of HPA. Then, 200 μl of 0.2 *M* perchloric acid and 4 ml of chloroform were added and the mixture was shaken vigorously and centrifuged again. The aqueous layer was further centrifuged at 156 600 *g* for 5 min at 4°C and, finally, an aliquot of 40 μl of the supernatant was analysed.

RESULTS

Chromatographic conditions

Six compounds were simultaneously separated using the mobile phase described above. Figs. 1 and 2 show the changes in the retention time of each com-

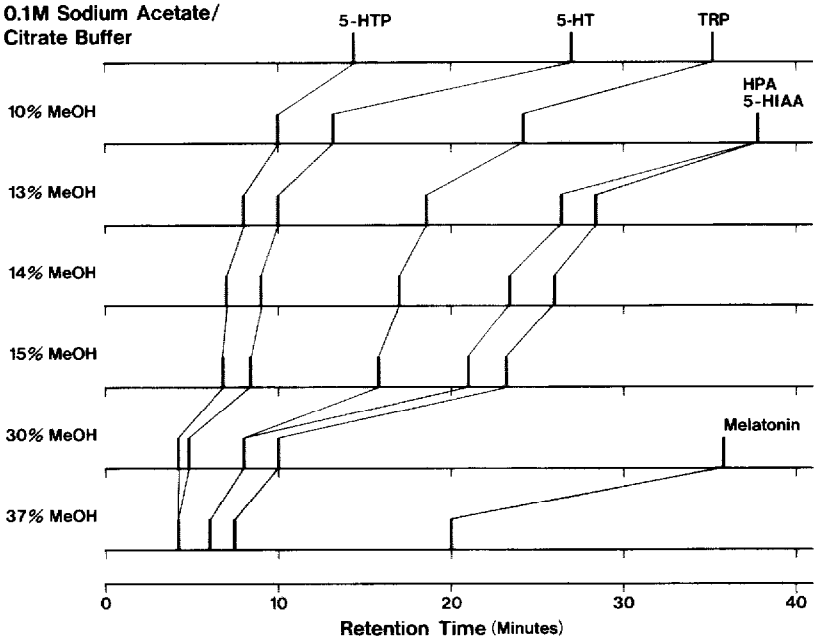

Fig. 1. Changes in the retention times of 5-HTP, 5-HT, TRP, HPA, 5-HIAA and melatonin with changes in the concentration of methanol (MeOH).

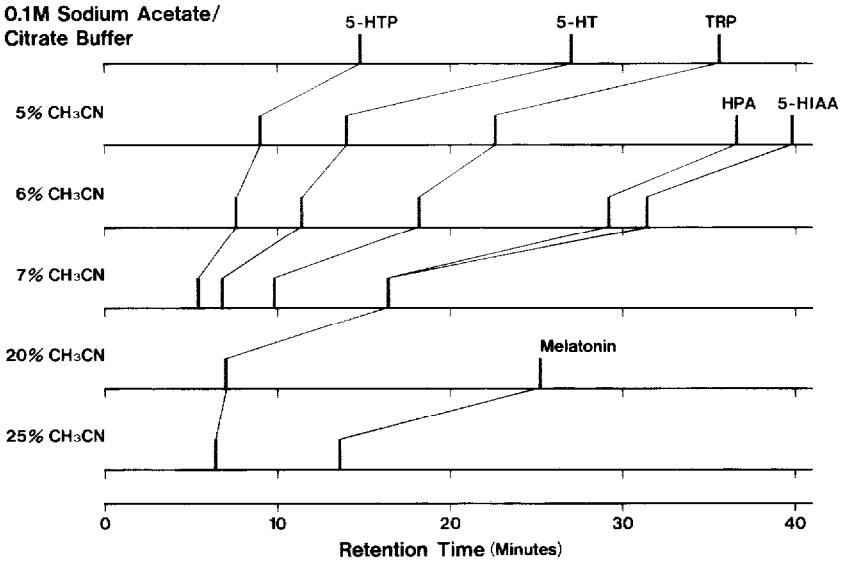

Fig. 2. Changes in the retention times of 5-HTP, 5-HT, TRP, HPA, 5-HIAA and melatonin with changes in the concentration of acetonitrile (CH_3CN).

pound with changes in the concentration of methanol from 10 to 37% and of acetonitrile from 5 to 25%. A 14% methanol concentration was the best for the separation of the peaks between 5-HIAA and HPA. A 5% acetonitrile concentration was optimal for the separation of each compound because of the baseline

resolution of all peaks. The determination of melatonin, on the other hand, required higher concentrations of methanol and acetonitrile for a peak to be obtained within 30 min. Finally, 20% acetonitrile was chosen because of the resolution of the peaks in plasma samples. Further, the separation of the metabolites was evaluated using the gradient elution system. However, no stable baseline could be obtained in the high-sensitivity range needed for plasma samples.

The relationship between potential and current for each compound was examined by sequentially changing the applied potential from 0.5 to 1.0 V (vs. Ag/AgCl). The three compounds with a 5-hydroxy group (5-HTP, 5-HT and 5-HIAA) were easily oxidized and reached a plateau at 0.7–0.8 V. The current applied to TRP, HPA and melatonin was increased starting from 0.85, 0.85 and 0.8 V. Finally, an applied potential of 0.9 V was chosen for the determination of 5-HTP, 5-HT, 5-HIAA and HPA, as it yielded a high sensitivity and a stable noise level and because the concentration of TRP in plasma was much higher than that of the 5-hydroxy compounds. For melatonin 0.95 V was chosen because of the high sensitivity needed for the analysis of plasma samples.

Linearity and sensitivity

The linearity of the calibration graph was studied by injecting 10, 15, 25, 50, 100, 200 and 500 pg of each standard solution to the HPLC system. The detector response to each compound was determined by measuring the height of their respective peaks and was found to be linear in the range tested. The correlation coefficient for each compound was higher than 0.9990. The limit of detection of each compound calculated at a signal-to-noise ratio of 3:1 is shown in Table I.

Precision and recovery

To estimate the recovery of each compound, maternal vein plasma was pooled and spiked with 10 ng/ml 5-HTP, 5-HT and 5-HIAA, 100 ng/ml TRP and HPA and 100 pg/ml melatonin standard solutions. The recovery and reproducibility of each compound through the entire procedure were determined by seven independent determinations under the same conditions (Table I). The practical de-

TABLE I

RECOVERIES FROM PLASMA AND DETECTION LIMITS OF 5-HTP, 5-HT, TRP, 5-HIAA, HPA AND MELATONIN ($n=7$)

Compound	Recovery		Detection limit (pg)
	Mean ± S.D. (%)	Inter-assay C.V. (%)	
5-HTP	99.2 ± 1.7	1.7	6.0
5-HT	100.0 ± 1.5	1.5	7.1
TRP	100.7 ± 5.7	5.7	17.5
5-HIAA	99.3 ± 1.5	1.5	7.6
HPA	99.0 ± 1.9	1.9	12.2
Melatonin	77.7 ± 3.5	4.4	15.0

tection limit for each compound in samples with the above coefficient of variation was less than 150 pg/ml for 5-HTP, 177.5 pg/ml for 5-HT, 190 pg/ml for 5-HIAA, 437 pg/ml for TRP and 19.3 pg/ml for melatonin.

Sample chromatogram

Fig. 3 shows the chromatogram of reference compounds and a sample of plasma from the umbilical artery at 39 weeks of gestation. The peaks of 5-HT, TRP, HPA

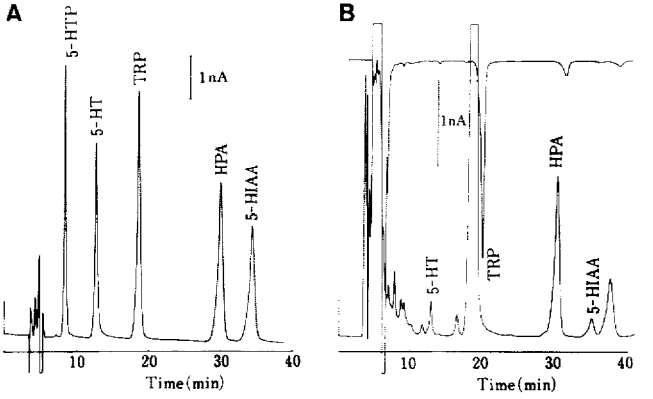

Fig. 3. Chromatograms of (A) standard 5-HTP (1.25 ng), 5-HT (1.25 ng), TRP (12.5 ng) and 5-HIAA (1.25 ng) solution and (B) a sample of umbilical artery plasma. Detector sensitivity: (A) 8 nA full scale; (B) 4 nA full scale (lower) and 40 nA full scale (upper). See text for chromatographic conditions.

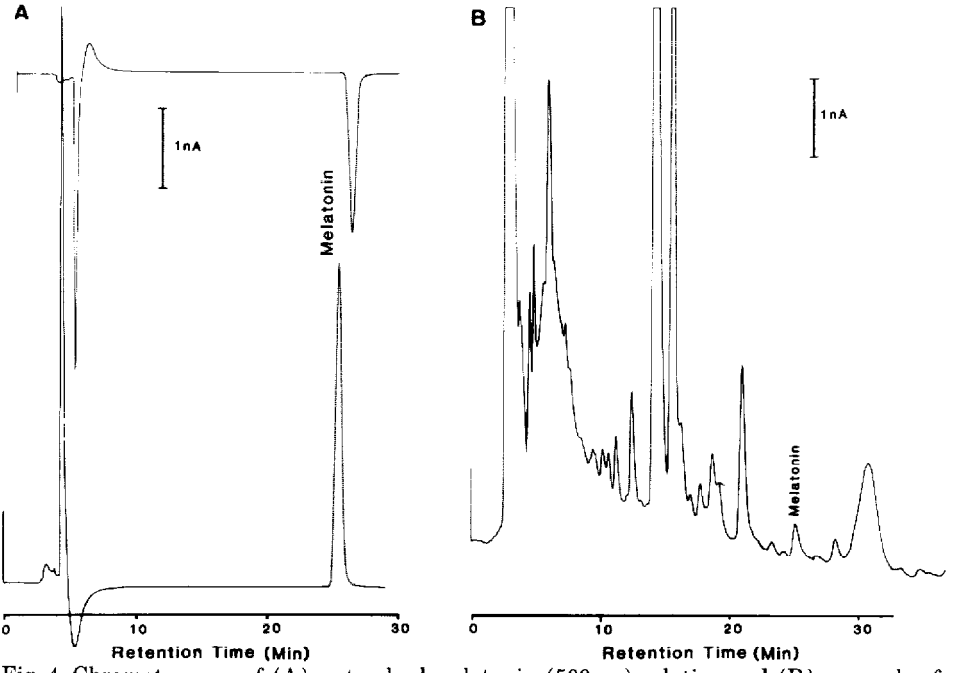

Fig. 4. Chromatograms of (A) a standard melatonin (500 pg) solution and (B) a sample of serum obtained at midnight from a woman. Detector senstivity: 8 nA full scale. See text for chromatographic conditions.

TABLE II

CONCENTRATIONS OF 5-HTP, 5-HT, TRP AND 5-HIAA IN PLASMA OF MATERNAL VEIN, UMBILICAL ARTERY AND UMBILICAL VEIN AT THIRD TRIMESTER

N.D. = not determined.

Compound	Concentration (ng/ml)		
	Maternal vein	Umbilical vein	Umbilical artery
5-HTP	N.D.	N.D.	N.D.
5-HT	1.61 ± 0.67 (n = 11)	2.12 ± 1.08 (n = 14)	2.66 ± 1.71 (n = 12)
TRP	6480.6 ± 2446.0 (n = 16)	12967.1 ± 2383.7 (n = 16)	12323.8 ± 2355.2 (n = 16)
5-HIAA	7.32 ± 2.95 (n = 16)	20.36 ± 6.32 (n = 16)	24.13 ± 5.04 (n = 16)

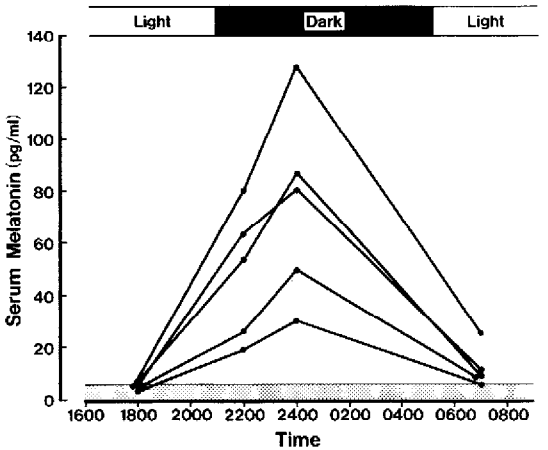

Fig. 5. Circadian changes of the individual plasma melatonin concentration in five healthy subjects with normal menstrual cycles.

and 5-HIAA were detected. However, the 5-HTP peak was not detected. Fig. 4 shows the chromatogram of a reference melatonin and a serum sample obtained at midnight from a woman. The melatonin peak was also observed. Table II gives the plasma concentrations of TRP and its metabolites in maternal vein, umbilical artery and umbilical vein plasma obtained by the present system. Fig. 5 shows the circadian changes in the individual plasma melatonin levels in five women. The plasma melatonin concentrations (mean ± S.D.) at 10 p.m., midnight and 7 a.m. were 48.7 ± 22.9, 75.2 ± 33.7 and 12.2 ± 7.0 pg/ml, respectively. The values at noon were too low to be detected by this system.

DISCUSSION

We prepared plasma with Na_2EDTA to prevent the gradual increase in plasma 5-HT levels after blood collection. No platelets were detected in plasma either

microscopically or thrombocytometrically, and 5-HT and 5-HIAA remained stable at 4 °C for at least 8 h after preparation. With regard to sensitivity, the detection limits of 5-HT and 5-HIAA were 177.5 and 190.0 pg/ml, respectively, which is sufficient for the clinical determination of pregnancy. Plasma melatonin has been determined so far either by radioimmunoassay [10] or by gas chromatography–mass spectrometry [11] because of its low levels. In 1980, Goldman et al. [12] reported the determination of melatonin by HPLC with electrochemical detection for the first time. However, the limit of detection was 50 pg for a signal-to-noise ratio of 3:1, which seems to be insufficient for the quantification of plasma melatonin. With the present method the detection limit of melatonin was 19.3 pg/ml, which is sufficient to detect plasma melatonin levels. Nocturnal plasma melatonin was also measured by the present method. The proposed method should, therefore, provide a valuable tool for the investigation of the pathophysiological role of 5-HT during pregnancy and of melatonin in the regulation of gonadal function.

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