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Determination of Serotonin and Melatonin in Rat Pineal Gland by High-Performance Liquid Chromatography with Ultraviolet and Fluorometric Dual Detection

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High-performance liquid chromatography (HPLC) of serotonin and melatonin was carried out on a reversed-phase column; the compounds were determined by fluorescence measurement with excitation and emission wavelengths of 285 and 345 nm, respectively. The indoles were clearly separated and eluted with a single mobile phase within 20 min. 4-Aminoantipyrine was used as an internal standard (detected by ultraviolet (UV) absorbance measurement). Satisfactory recovery of serotonin and melatonin was obtained by using this dual detection system (UV and fluorescence). The detection limits of the indoles were all in the low picogram range.

The proposed method was applied to the determination of serotonin and melatonin in rat pineal gland. A distinct variation between light and dark periods was found in both serotonin and melatonin levels in the rat pineal gland.

Keywords——native fluorescence; dual detection system; high-performance liquid chromatography; melatonin; serotonin; rat pineal gland

Introduction

It is well known that several indole compounds, which exert neurochemically important physiological actions, exist in the pineal gland.[1] Among the pineal indoles, serotonin, which is a neurotransmitter in the central nervous system and is considered to be related to several psychiatric disorders,[2] is found abundantly in the pineal tissue. On the other hand, melatonin, one of the pineal indoles, mediates the action of the pineal gland on reproductive function.[3] Quantitative measurements of these indoles in the pineal gland of mammals (including human) have mainly depended on bioassay,[4] gas chromatography-mass spectrometry (GC-MS),[5] and radioimmunoassay.[6] All these methods have particular merits and demerits in respect of sensitivity, selectivity, specificity or convenience. Recently, high-performance liquid chromatography (HPLC) on a reversed-phase column has been developed as a powerful technique for separating related compounds.[7] We have now developed an HPLC procedure with a combined fluorometric/ultraviolet (UV) spectrometric detection system and a single isocratic mobile phase to detect and determine indole compounds, using 4-aminoantipyrine as an internal standard.

This paper also describes an application of the procedure for the determination of serotonin and melatonin in the rat pineal gland.

Experimental

Reagents and Solutions——Serotonin, melatonin and 5-methoxytryptamine were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Stock solutions (10 mg/100 ml) of each indole compound were prepared by dissolving each substance in 0.1 M perchloric acid containing 0.1% ascorbic acid, and diluting the solution to the appropriate concentration prior to use. The solutions were stored at 4 °C. 4-Aminoantipyrine (4-AA, internal standard) was

purchased from Wako Pure Chemical Co. (Osaka, Japan). 4-AA solutions as an internal standard were prepared by dissolving 4-AA in 0.1 M perchloric acid containing 0.1% ascorbic acid to give concentrations of 50 mg/100 ml (internal standard A solution) for serotonin and 14 mg/100 ml (internal standard B solution) for melatonin determination. The solutions were shielded from light and stored at 4 °C.

Chromatography——Chromatography was performed on a Hitachi HPLC model 635 (Tokyo, Japan) equipped with an RF-530 fluorescence spectromonitor (Shimadzu Seisakusho, Kyoto, Japan) and a UV monitor (UV-8, Toyo Soda Co., Tokyo, Japan). For detection of serotonin and melatonin, an excitation wavelength of 285 nm and emission wavelength of 345 nm were used. 4-AA was monitored with the UV detector at 248 nm. The column used was a Zorbax ODS (5 μm, 250 mm × 4.6 mm i.d.) with a built-in water jacket, controlled at 38 °C. The mobile phase was 0.01 M acetate buffer (pH 4.25)–methanol (65 : 35) mixture, and the flow rate was 1.2 ml/min.

Sample Preparations——Male Wistar rats (weighing 300—400 g, $n=15$) were maintained with a 12/12 h cycle of light and darkness for two weeks prior to the investigation. The rats were sacrificed at midnight (dark period) or 9 o'clock in the morning (light period), the pineal was rapidly removed and placed in a 10 ml glass-stoppered conical centrifuge tube chilled in ice, and then 100 μl of 0.1 M perchloric acid containing 0.1% ascorbic acid was added. The pineal tissue was disrupted with a sonicator for 1 min, and 10 μl of the homogenate (one-tenth volume of a pineal gland homogenate) was used for the serotonin assay. The remaining 90 μl of pineal homogenate was used for the melatonin assay. Forty μl of internal standard A solution was added to the homogenate for the determination of serotonin, and 10 μl of internal standard B solution was added for melatonin. Each mixture was centrifuged at 1500 *g* for 5 min at 0 °C. After centrifugation, the supernatant was filtered through a Column Guard® (Millipore Co., Ltd.). A 2 or 5 μl portion of the clear filtrate was injected into the HPLC for the determination of serotonin, while a 50 μl portion of the filtrate was used for the determination for melatonin.

Identification of Melatonin and Serotonin——The peak corresponding to melatonin (t_R 14.3 min) on the chromatogram of the pineal extract was identified by thin-layer chromatography (TLC) on Kieselgel 60 F-254 (5 × 20 cm, Merck) with $CHCl_3$–MeOH (9 : 1).[8] The spot was visualized by UV irradiation. Eluate corresponding to the peak at t_R 14.3 min was separately collected and concentrated *in vacuo*. The concentrate was subjected to TLC along with authentic melatonin, and the *Rf* values were compared. Next, the concentrate (0.5 ml) containing melatonin of three pineals and authentic melatonin were individually hydrolyzed with 0.3 ml of 20% HCl at 100 °C for 40 min. Each mixture was neutralized with 1 N NaOH, and then concentrated *in vacuo*. The *Rf* values on TLC of the neutralized hydrolysates were compared with that of 5-methoxytryptamine. For the identification of serotonin, the eluate at around t_R 3.0 min was also concentrated to 0.5 ml *in vacuo*. The concentrate was subjected to chromatography again with 0.01 M acetate buffer (pH 4.50)–acetonitrile (90 : 10) mixture as the mobile phase. The chromatographic behavior of the concentrate was compared with that of authentic serotonin.

Examination of the Usefulness of the Dual Detection System——A 0.1 M perchloric acid solution containing melatonin (10 ng/ml) and 4-AA (14 μg/ml) was freshly prepared. A 50 μl aliquot was injected into the HPLC three times a day before, during, and after the chromatographic analysis of pineal extracts for 20 d. The ratio of the peak height of melatonin to that of the internal standard, 4-AA, was calculated.

Results and Discussion

Figure 1 shows chromatograms of a mixture containing authentic indoles and 4-AA used as an internal standard. Clear separation of the indole compounds was obtained with a single mobile phase, whereas several mobile phases have been required in conventional HPLC. The detection limit based on a signal-to-noise ratio of 2 : 1 was 10 pg of serotonin and 30 pg of melatonin. Thus, pg quantities of both indoles can be detected by using a fluorometric detector.

The use of an internal standard for analytical measurement is generally adopted in order to correct for losses during the extraction procedure. However, little attempt has previously been made to find suitable standards in chromatographic procedures for the determination of pineal indole amines. We therefore examined various indole-related compounds other than serotonin and melatonin. However, an appropriate fluorescent internal standard could not be found, because many melatonin-related compounds have chromatographic properties similar to those of the target compound, or are not commercially available. We next investigated UV-absorbing substances, and found that 4-AA, which is not fluorescent, did not interfere with the separation of serotonin and melatonin, and was not adsorbed by pineal tissue homogenate ($100.5 \pm 1.2\%$ recovery). Thus, 4-AA may be applicable as an internal standard for the determination of serotonin and melatonin if a dual detection system, fluorometer and UV

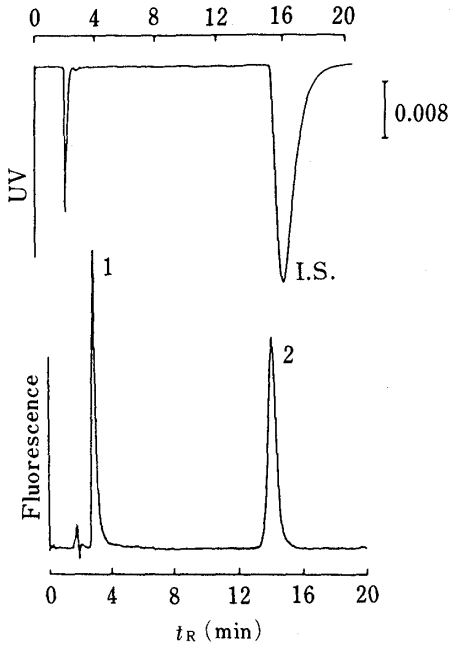

Fig. 1. Chromatograms of an Authentic Mixture of Indoles and 4-Aminoantipyrine (Internal Standard)

Peak 1, serotonin; 2, melatonin; I.S., 4-aminoantipyrine (4-AA).

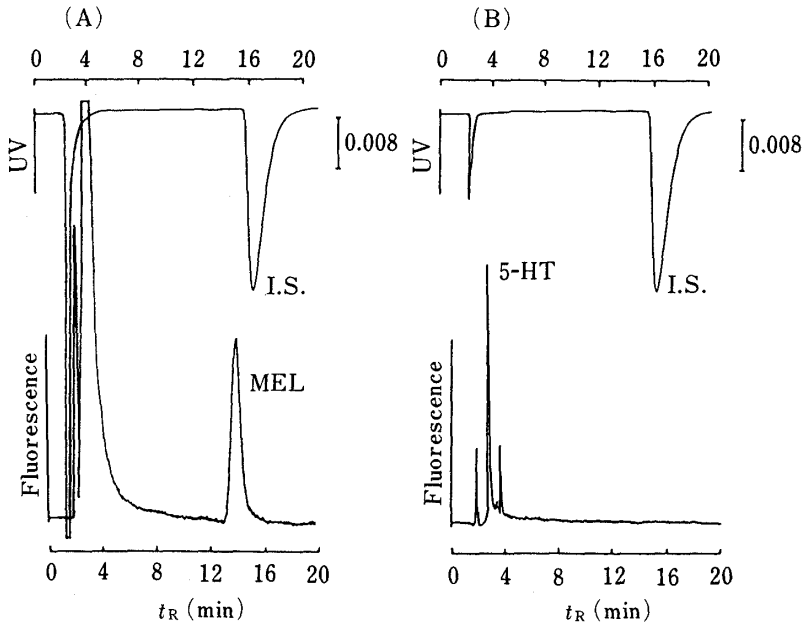

Fig. 2. Chromatograms of Rat Pineal Gland Extract

Male Wistar rats were maintained on a light-dark 12/12 h cycle for two weeks before sacrifice. The lights were turned on at 7:00 a.m. and off at 7:00 p.m. The pineal gland was removed at 0:00 a.m. (dark period).

(A) Determination of melatonin (MEL) in a rat pineal gland.

(B) Determination of serotonin (5-HT) in a rat pineal gland.

spectrometer, can be used. We therefore investigated the parallelism between fluorometry and UV spectrometry results. A 50 μl portion of a mixture containing known amounts of melatonin and 4-AA was injected into the HPLC three times a day, and the ratio of peak height of melatonin to that of 4-AA was calculated. The experiment was repeated daily for 20 d in order to check between-day variations. The coefficient of variation (CV) value of 0.68% ($n=60$) thus obtained suggests that the dual detection system employed in the present study is effective.

In order to determine the amounts of indoles in the pineal gland by the proposed method,

calibration curves for serotonin and melatonin were prepared by plotting the ratios of peak heights of serotonin and melatonin to that of 4-AA. The equations representing the calibration curves were $y=0.00728x+0.00535$ ($r=0.998$) between 10 and 600 pg of serotonin, and $y=0.00196x+0.05027$ ($r=0.998$) between 30 and 1000 pg of melatonin.

Figure 2 shows chromatograms of the extract obtained from rat pineal gland removed at midnight (dark period) for the determination of melatonin (Fig. 2A) and serotonin (Fig. 2B). A peak corresponding to melatonin can be seen at t_R 14.3 min in the chromatogram in Fig. 2(A). For the identification of this peak, the eluate around 14.3 min retention time was collected and concentrated to a small volume *in vacuo*. The concentrate was compared with authentic melatonin in terms of *Rf* value of TLC. The *Rf* value of the concentrate was 0.68, and agreed well with that of melatonin. Further, the concentrate and melatonin were each hydrolyzed with hydrochloric acid and the *Rf* values on TLC of the hydrolysates were compared with that of 5-methoxytryptamine. The hydrolysate of the eluate corresponding to t_R 14.3 min and the hydrolysate of melatonin each showed a single spot on TLC at the same *Rf* value (0.10) as that of 5-methoxytryptamine. On the basis of these experimental results, the peak corresponding to t_R 14.3 min was identified as melatonin. Identification of the peak at t_R 3.0 min in Fig. 2(B) was also carried out by rechromatography with a different solvent system. The retention time of the peak with a different mobile phase was 4.6 min, and agreed well with that of authentic serotonin. The peak was therefore attributed to serotonin.

Recoveries of melatonin and serotonin added to rat pineal glands are given in Tables I and II, respectively. Five rat pineals removed at 9 o'clock in the morning (light period) were combined and homogenized in 0.1 M perchloric acid with the sonicator. The homogenate was divided into two equal parts. A portion of perchloric acid solution containing melatonin was added to half of the homogenate, while as a control an equal volume of perchloric acid solution alone was added to the other half, and both were mixed well. The mixtures were each centrifuged at 1500 *g* for 5 min at 0 °C. The supernatants were filtered through a Column Guard® and portions of the filtrate were directly injected into the HPLC. The amount of melatonin added was 160 or 800 pg per pineal. The melatonin contents were calculated by subtracting the amount originating in the tissue (control value in Table I) from the total amount. Recovery tests of serotonin were also carried out in accordance with the procedures described above. The recoveries were all satisfactory, regardless of the amounts of melatonin and serotonin added to the pineal extract. The combination of low-speed centrifugation (1500 *g*) and filtration through a Column Guard® was thus an effective sample preparation procedure for these indole-related compounds. To investigate the adsorption of the indoles

TABLE I. Analytical Recovery of Melatonin Added to Rat Pineal Gland[a]

Melatonin				
Added[b] (pg)	Found[c] (pg)	Recovery[d]		CV (%)
		(pg)	(%)	
0 (Control)	322.2±5.7			
160	482.3±4.2	160.3±4.0	100.1±2.6	2.6
0 (Control)	354.6±5.6			
800	1152.9±9.9	798.2±9.8	99.8±1.3	1.3

a) Five pineals removed at 9 o'clock in the morning were combined and homogenized. A recovery test was carried out using exactly half of the homogenate, and the other half was used as the control. *b*) Amount of melatonin added to a rat pineal gland. *c*) Melatonin content per rat pineal gland. Each value represents the mean±S.D. of five measurements. *d*) Each value represents the mean±S.D. of five measurements.

TABLE II. Analytical Recovery of Serotonin Added to Rat Pineal Gland[a]

Serotonin				
Added[b] (ng)	Found[c] (ng)	Recovery[d] (ng)	Recovery[d] (%)	CV (%)
0 (Control)	158.1±3.9			
50	206.3±5.1	48.2±1.3	96.4±2.6	2.7
0 (Control)	157.9±3.8			
200	354.4±8.0	196.4±8.0	98.2±4.0	4.1

a, d) See Table I. *b*) Amount of serotonin added to a rat pineal gland. *c*) Serotonin content per rat pineal gland. Each value represents the mean±S.D. of five measurements.

TABLE III. Contents of Serotonin and Melatonin in Rat Pineal Gland

Time	Period	ng/Pineal gland[a]	
		Serotonin	Melatonin
0:00	Dark	47.1±16.4	1.65±0.15
9:00	Light	125.5±27.5	0.31±0.08

a) Each value represents the mean±S.D. of five measurements.

and 4-AA on the Column Guard,® the amounts of these compounds were determined before and after passage of solutions containing known concentrations of the compounds through it. It became clear that none of the indoles or 4-AA was adsorbed on the Column Guard.® The proposed technique could easily afford a clear supernatant in a short time without using a high-speed centrifugation procedure.

The contents of serotonin and melatonin found in the rat pineal gland are listed in Table III. Pineal serotonin contents were high in the light period compared with the dark period, while melatonin contents showed an opposite pattern.[9] It is known that melatonin contents in the pineal vary depending on the age and body weight of animals.[10] In this investigation using male rats at 14 weeks of age (weighing 300—400 g), the contents of serotonin and melatonin in rat pineal were similar to those given previously.[7a,c]

In conclusion, we have developed a method for the determination of serotonin and melatonin in rat pineal gland by fluorometric HPLC using an ultraviolet-absorbing substance as an internal standard. From the results described above, the proposed method is as sensitive as an electrochemical detection system or radioimmunoassay, which is usually thought to be highly sensitive.

Thus, the proposed method is rapid, simple, and sensitive, and allows the simultaneous determination of serotonin and melatonin in the pineal gland.

References

1) J. Axelrod, *Science*, **184**, 1341 (1974); R. J. Reiter and M. K. Vaughan, *Life Sci.*, **21**, 159 (1977); R. J. Reiter, B. A. Richardson, and T. S. King, "The Pineal Gland," ed. by R. Relkin, Elsevier, Inc., New York, 1983, pp. 151—199.
2) B. S. Meldrum, "Disorders of Neurohumoural Transmission," ed. by T. J. Crow, Academic Press, London, 1982, pp. 220—222; T. J. Crow, *ibid.*, pp. 292—309.

3) L. Tamarkin, W. K. Westrom, A. I. Hamill, and B. D. Goldman, *Endocrinol.*, **99**, 1534 (1976); D. J. Kennaway, T. A. Gilmore, and R. F. Seamark, *ibid.*, **110**, 1766 (1983).
4) C. L. Ralph and H. J. Lynch, *Gen. Comp. Endocrinol.*, **5**, 334 (1970).
5) F. Cattabeni, S. H. Koslow, and E. Costa, *Science*, **178**, 166 (1972); B. W. Wilson, W. Snedden, R. E. Silman, I. Smith, and P. Mullen, *Anal. Biochem.*, **81**, 283 (1977).
6) D. J. Kennaway, R. G. Frith, G. Philipou, C. D. Matthews, and R. F. Seamark, *Endocrinol.*, **101**, 119 (1977); M. Wilkinson, J. Arendt, J. Bradtke, and D. deZeigler, *J. Endocrinol.*, **72**, 243 (1977); S. Steinlechner, T. H. Champney, M. L. Houston, and R. J. Reiter, *Proc. Soc. Exp. Biol. Med.*, **175**, 93 (1984).
7) *a*) I. N. Mefford and J. D. Barchas, *J. Chromatogr.*, **181**, 187 (1980); *b*) G. M. Anderson, J. G. Young, D. K. Batter, S. N. Young, D. J. Cohen, and B. A. Shaywitz, *ibid.*, **223**, 315 (1981); *c*) G. M. Anderson, J. G. Young, D. J. Cohen, and S. N. Young, *ibid.*, **228**, 155 (1982).
8) J. Arendt, *J. Neural Transm., Suppl.*, **13**, 265 (1978).
9) W. B. Quay, *Gen. Comp. Endocrinol.*, **3**, 473 (1963); H. J. Lynch, *Life Sci.*, **10**, 791 (1971); S. N. Young and G. M. Anderson, *Neuroendocr.*, **35**, 463 (1982).
10) R. J. Reiter, "The Pineal Gland and Its Endocrine Role," ed. by J. Axelrod, F. Fraschini, and G. P. Velo, Plenum Press, New York, 1983, pp. 227—241; S. F. Pang, F. Tang, and P. L. Tang, *J. Exp. Zool.*, **229**, 41 (1984).