Journal of Chromatography, 381 (1986) 21—28
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3187

VARIATION OF MELATONIN AND SEROTONIN CONTENT IN RAT PINEAL GLAND WITH SEX AND OESTROUS PHASE DIFFERENCE DETERMINED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORIMETRIC DETECTION

HIROYUKI WAKABAYASHI* and KENJI SHIMADA

Niigata College of Pharmacy, 5829 Kamishin'ei-cho, Niigata, 950-21 (Japan)

and

YOSHIO AIZAWA

Nakano Egota Hospital, 4-19-9, Egota, Nakanoku, Tokyo, 165 (Japan)

(First received January 29th, 1986; revised manuscript received March 26th, 1986)

SUMMARY

Simultaneous determination of melatonin and serotonin in rat pineal gland is described using reversed-phase high-performance liquid chromatography with fluorimetric detection. These indoles were analysed isocratically within 15 min. In this work, veratric acid (3,4-dimethoxybenzoic acid), which has fluorescence characteristics (λ_{ex} = 290 nm, λ_{em} = 350 nm) around the wavelength of native fluorescence of melatonin (λ_{ex} = 285 nm, λ_{em} = 345 nm), was used as an internal standard. This method was applied to the determination of melatonin and serotonin in male and female rat pineal gland. No significant differences between the two groups were observed in the pineal melatonin and serotonin contents. The pineal melatonin and serotonin contents were compared with the oestrous and the di-oestrous phases of female rats. They were not widely different from each other.

INTRODUCTION

Melatonin and serotonin in the pineal gland are biosynthesized from tryptophan, and the concentrations of these indoles are closely related to the circadian rhythms in the pineal gland [1—3]. Pineal melatonin content is high during the night and low during the day, while the levels of serotonin exhibit

0378-4347/86/$03.50

the opposite behaviour. Melatonin and serotonin exert important physiological actions [4—8], and melatonin is particularly well known to block ovulation and inhibit the incidence of the oestrous phase in the rat [9, 10].

Various methods have been applied for the determination of melatonin and its related indoleamines in pineal gland [11—17]. High-performance liquid chromatography (HPLC) has been used for the determination of pineal indoles, because of the simplicity of sample preparation and the capability of simultaneous determination of the indole compounds [18—20]. The authors have recently reported an HPLC method with dual detection for the determination of melatonin and serotonin in rat pineal gland [21]. In this method, 4-aminoantipyrine was adopted as an internal standard to improve the analytical precision.

The present report describes the HPLC analysis of melatonin and its related pineal indoleamines with fluorimetric detection. In this method veratric acid, which has fluorescence characteristics similar to the native fluorescence of melatonin, was used as an internal standard instead of 4-aminoantipyrine. This use of the internal standard increased the precision of the assay.

This paper also describes the determination of indole compounds related to melatonin in rat pineal tissue. The difference between male and female rats was investigated, together with the effect of the oestrous cycle in female rats.

EXPERIMENTAL

Reagents and solutions

Melatonin, serotonin, N-acetylserotonin, 5-methoxytryptophol, 5-hydroxytryptophol and 5-methoxyindoleacetic acid were purchased from Sigma (St. Louis, MO, U.S.A.). Stock solutions of each compound (10 mg per 100 ml) were prepared in 0.1 *M* perchloric acid containing 0.1% ascorbic acid, and diluted to appropriate concentrations prior to use. The stock solutions were stored at 4°C. Veratric acid (internal standard) was purchased from Aldrich (Milwaukee, WI, U.S.A.). As internal standard solution A for melatonin determination, veratric acid was dissolved in 0.1 *M* perchloric acid containing 0.1% ascorbic acid at a concentration of 3.6 mg per 100 ml and diluted 100-fold with the solvent described above prior to use. As internal standard solution B for serotonin determination, veratric acid solution (17.5 mg per 100 ml) was prepared and diluted in the same manner as solution A. Both internal standard solutions were stored at 4°C.

Chromatography

Chromatography was performed by a Hitachi Model 635 (Japan) high-performance liquid chromatograph equipped with an RF-530 fluorescence spectromonitor (Shimadzu Seisakusho, Japan). Excitation and emission wavelengths were set at 285 nm and 345 nm, respectively. The analytical column packed with Zorbax ODS (250 mm × 4.6 mm I.D., 5 μm average particle size, DuPont, Wilmington, DE, U.S.A.) was used at 38°C. The mobile phase was a mixture of 0.01 *M* acetate buffer (pH 4.25) solution and methanol (65:35). The solution was filtered through a microfilter (0.4 μm) and degassed. The flow-rate was 1.2 ml/min.

Sample preparation

Male and female Wistar rats of ca. 200 g in weight were maintained under a cycle of light and darkness for 12 h (lights on at 7:00 a.m.) for two weeks prior to investigation. The animals were given access to food and water ad libitum. The oestrous cycles of the females were determined daily by vaginal smear tests at 8:30 a.m. The rats were sacrificed by decapitation at 9:00 a.m. and the pineal glands were rapidly removed. The pineal glands were placed in 10-ml glass-stoppered conical centrifuge tubes chilled in ice, and then 100 μl of ice cold 0.1 *M* perchloric acid containing 0.1% ascorbic acid were added. The pineal tissue was disrupted by a sonicator for 1 min and 90 μl of the homogenate (nine tenths of the pineal homogenate) were used for the melatonin assay. A 10-μl aliquot of internal standard solution A was added to the homogenate for the determination of melatonin. For the serotonin assay, 10 μl of pineal homogenate (one tenth of the pineal homogenate) were used, and 40 μl of internal standard solution B were added to the homogenate for the serotonin assay. Both mixtures were centrifuged at 1500 *g* for 5 min at 0°C and the supernatant was filtered through a Column Guard® (Millipore). A 50-μl aliquot of the clear filtrate was injected into the chromatograph for the determination of melatonin, whereas a 5-μl portion of the filtrate was used for the determination of serotonin.

RESULTS AND DISCUSSION

For the determination of catecholamines by HPLC, an internal standard has usually been used in many previous investigations [22—25], but the same is not true for assays of melatonin and its related indoleamines. In order to reduce the errors during the extraction procedure and to increase analytical precision, we recently reported an HPLC method with dual detection using a UV-absorbing substance as internal standard for the determination of rat pineal indoleamines [21]. To simplify further the analytical procedure in the previous method, we examined the use of a suitable internal standard having fluorescence characteristics similar to indoleamines.

Consequently, veratric acid (3,4-dimethoxybenzoic acid) was adopted as an internal standard. Since veratric acid fluoresces at an excitation wavelength of 290 nm and an emission wavelength of 350 nm, the compound was detectable at the wavelength of native fluorescence of melatonin (λ_{ex} = 285 nm, λ_{em} = 345 nm). Furthermore, the retention behaviour of this substance differed from those of pineal indoleamines. By the adoption of fluorescent veratric acid as internal standard, the chromatographic determination was simpler than in the former method [21] for melatonin and its related compounds.

Fig. 1 shows the isocratic separation of a mixture of authentic melatonin and its related indoles, and veratric acid. The detection limit, based on a signal-to-noise ratio of 2:1, was 10 pg of serotonin, 30 pg of melatonin, 10 pg of 5-hydroxytryptophol, 15 pg of N-acetylserotonin, 45 pg of 5-methoxyindole-acetic acid and 25 pg of 5-methoxytryptophol.

The above results show that the proposed assay for melatonin and its related indoles using fluorimetric detection is as sensitive as using the electrochemical method of detection reported by Anderson et al. [19].

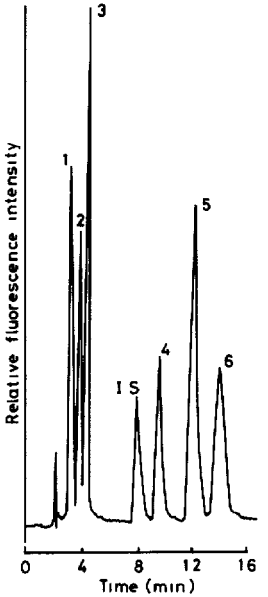

Fig. 1. Chromatogram of authentic indoleamines and veratric acid (internal standard). Chromatographic conditions: mobile phase 0.01 *M* acetate buffer—methanol, pH 4 25 (65·35), flow-rate, 1.2 ml/min. Peaks: 1 = serotonin; 2 = 5-hydroxytryptophol; 3 = N-acetylserotonin, 4 = 5-methoxyindoleacetic acid; 5 = 5-methoxytryptophol; 6 = melatonin (MEL); I.S. = veratric acid, internal standard.

In order to determine the amounts of pineal melatonin and serotonin that exert neurochemically important actions, calibration curves for melatonin and serotonin were drawn by plotting the peak-height ratios for melatonin and serotonin to that of the internal standard. The equations representing calibration curves were $y = 0.00194x + 0.00426$ ($r = 0.998$) between 30 and 1000 pg for melatonin, and $y = 0.00175x - 0.00118$ ($r = 0.998$) between 10 and 1000 pg for serotonin. The within-day coefficients of variation of the peak-height ratios for authentic melatonin and serotonin to that of the internal standard were $< 3\%$. In addition to melatonin and serotonin, calibration curves of other melatonin-related indoles were similarly obtained. The regression equations were $y = 0.00243x - 0.00536$ ($r = 0.998$) between 10 and 1000 pg for 5-hydroxytryptophol, $y = 0.00188x + 0.0014$ ($r = 0.993$) between 15 and 1000 pg for N-acetylserotonin, $y = 0.00148x - 0.0006$ ($r = 0.988$) between 50 and 1000 pg for 5-methoxyindoleacetic acid, and $y = 0.00272x - 0.00039$ ($r = 0.998$) between 25 and 1000 pg for 5-methoxytryptophol.

The chromatograms of the extract obtained from rat pineal tissue are shown in Figs. 2 and 3. Fig. 2 shows the chromatograms of serotonin determined in the pineal specimen taken in the light period (9:00 a.m.) (A) and in the dark period (0:00 a.m.) (B), respectively. It is well known that serotonin contents of rat pineal tissue are high in the light period compared with the dark period. These results are in agreement with those reported by Quay [1] and Klein and Weller [3]. Fig. 3 shows a typical chromatogram for the determination of melatonin in rat pineal tissue removed at 9:00 a.m. (light period). It has been reported that the circadian rhythm in pineal melatonin shows an opposite

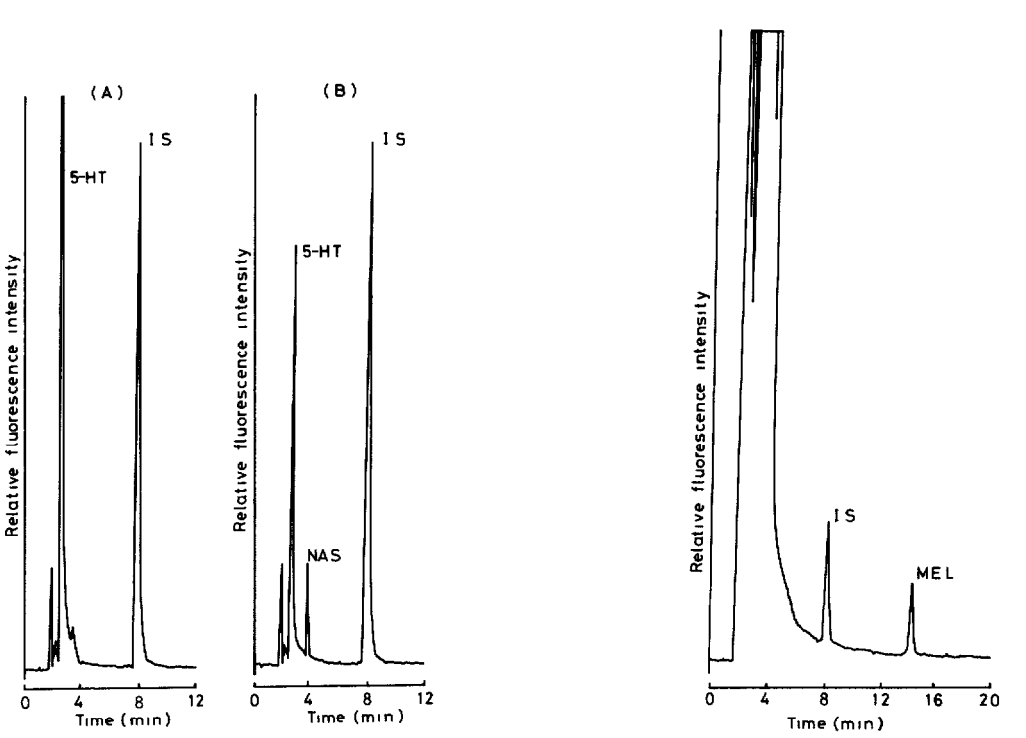

Fig. 2. Chromatograms of rat pineal gland extract. Wistar rats were maintained on a light—dark (12—12 h) cycle for two weeks before use. (A) Extract of pineal gland removed at 9:00 a.m. (light period); (B) extract of pineal gland removed at 0:00 a.m. (dark period). Chromatographic conditions: see Fig. 1. Peaks: 5-HT = serotonin; NAS = N acetylserotonin; I.S. = veratric acid, internal standard.

Fig. 3. Chromatogram of rat pineal gland extract. Pineal gland was removed at 9:00 a.m. (light period). Chromatographic conditions: see Fig. 1. Peaks: MEL = melatonin; I.S. = veratric acid, internal standard.

pattern to that of serotonin [1—3, 26]. Using the proposed HPLC method, rat pineal melatonin was measurable even in the light period.

In previous HPLC methods [18—20], several mobile phases were required for the separation of pineal melatonin and serotonin. As described under *Sample preparation*, the homogenate of a pineal was divided into two portions for melatonin and serotonin assays, respectively. Using the improved extraction procedure in the proposed HPLC method, pineal indoleamines were clearly separated with an isocratic mobile phase.

By a combination of the improved extraction procedure and the separation method by a single mobile phase, the HPLC conditions were stable and rapid determination was achieved.

Identification of melatonin and serotonin peaks on the chromatograms of the rat pineal sample was made as in the previous paper [21]. In Fig. 2B, another peak besides the serotonin peak appeared at a retention time (t_R) of 4.0 min, indicating the existence of N-acetylserotonin produced by N-acetylation of serotonin, which is a precursor of melatonin. It has been shown that pineal N-acetylserotonin contents are lower during the day time and 10—30

times higher during the night time [3, 26]. The circadian variation of N-acetylserotonin in rat pineal tissue was also supported by the results in Fig. 2A and B. In the dark period (0:00 a.m.), the values obtained for N-acetylserotonin in rat pineal tissue were 6.5 ± 2.4 ng per pineal gland (n = 10). The concentration range for pineal N-acetylserotonin is similar to that reported by Anderson et al. [20] and Pang et al. [27].

Recovery results of melatonin and serotonin added to rat pineal glands are given in Tables I and II, respectively. The amounts of melatonin added were 0.2 and 2.0 ng per pineal gland, individually (Table I). The recovery rates of melatonin were calculated by subtracting the values of the unspiked sample (control) from those of the spiked sample, and the recovery rates of both added groups were more than 95% (coefficient of variation < 2%, n = 5). The recovery of serotonin was also determined using the procedures described above, and the recovery rates of serotonin are shown in Table II. The results were all quantitative, regardless of the amounts of serotonin added to the pineal homogenates.

The proposed HPLC method was applied to the determination of melatonin and serotonin in a pineal gland of the Wistar rat.

Fig. 4 shows the conents of melatonin and serotonin in male and female rat pineal glands that were removed at 9:00 a.m. (light period). Female rats were

TABLE I

ANALYTICAL RECOVERY OF MELATONIN ADDED TO RAT PINEAL GLAND

Each value represents the mean ± S.D. (n = 5).

Added* (ng)	Found** (ng)	Recovery		Coefficient of variation (%)
		(ng)	(%)	
0 (control)	0.22 ± 0.003			
0.2	0.41 ± 0.004	0.19 ± 0.004	95.0 ± 2.0	2
2.0	2.21 ± 0.04	1.99 ± 0.04	99 5 ± 2 0	2

*Amount of melatonin added to a rat pineal gland
**Melatonin content per rat pineal gland.

TABLE II

ANALYTICAL RECOVERY OF SEROTONIN ADDED TO RAT PINEAL GLAND

Each value represents the mean ± S.D. (n = 5).

Added* (ng)	Found** (ng)	Recovery		Coefficient of variation (%)
		(ng)	(%)	
0 (control)	120.9 ± 2.4			
50	169 2 ± 2.5	48.3 ± 2.5	96.6 ± 4.9	5
200	316.2 ± 6.1	195.3 ± 6.1	97.7 ± 3.1	3

*Amount of serotonin added to a rat pineal gland.
**Serotonin content per rat pineal gland.

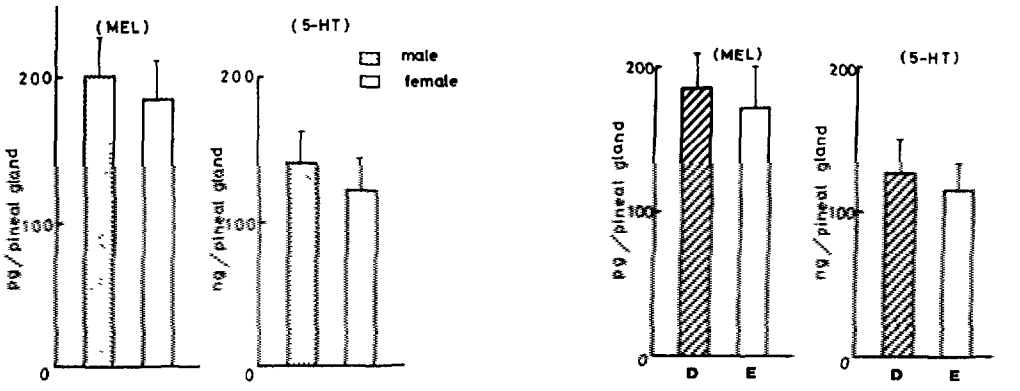

Fig. 4. Pineal melatonin and serotonin contents in male and female rats. Pineal gland was removed at 9:00 a.m. (light period). Each column shows the mean ± S.D. (n = 10).

Fig. 5. Rat pineal melatonin and serotonin contents in the oestrous (E, □) and the di-oestrous (D, ▨) phase. The oestrous cycle was determined by vaginal smear test at 8:30 a.m. and the pineal was removed at 9:00 a.m. (light period). Each column shows the mean ± S.D. (n = 10).

used without determining the oestrous cycles. Melatonin contents in male rat pineal gland were 200.8 pg per pineal gland (n = 10), while in female rat these were 179.0 pg per pineal gland (n = 10). Pineal serotonin contents were 141.3 ng per pineal gland for male (n = 10) and 121.4 ng per pineal gland for female rats (n = 10). No significant differences between male and female rats were noted in pineal contents of both melatonin and serotonin ($P < 0.05$). Similar results were reported for the pineal gland of the Syrian Hamster [28].

Fig. 5 shows the melatonin and serotonin contents in pineal gland at the oestrous and the di-oestrous phases in female rats. Melatonin contents were 183.3 pg per pineal gland (n = 10) at the di-oestrous and 170.7 pg per pineal gland (n = 10) at the oestrous phase. Pineal serotonin contents at the di-oestrous and the oestrous phases were 126.2 and 118.1 ng per pineal gland, respectively. There were also no differences between the oestrous and the di-oestrous phases in pineal melatonin and serotonin contents ($P < 0.05$). It has been reported that the ovulation and the incidence of the oestrous phase in female rats were inhibited by melatonin administration [9, 10]. However, the actions of melatonin could not be inferred from the endogenous melatonin levels in pineal gland. Melatonin is biosynthesized from N-acetylserotonin by hydroxyindole-O-methyl transferase (HIOMT) [29], which exists only in the pineal gland. Wurtman et al. [30] reported that the activity of HIOMT in the rat pineal gland during the di-oestrous phase is two to three times higher than that of the oestrous phase.

It is thus expected that the contents of pineal melatonin in the di-oestrous phase will probably be higher than that of the oestrous phase. However, our results (Fig. 5) do not show any significant differences in melatonin contents between the two phases of the oestrous cycle.

In conclusion, for the small amounts of melatonin present during the light period, the proposed HPLC method with fluorimetric detection is sufficiently sensitive for the determination of pineal indoleamines, as shown in Figs. 4 and 5.

ACKNOWLEDGEMENTS

The authors wish to thank Mr. Yoshiteru Okada and Mr. Kazumasa Yuasa for their assistance in the experimental work.

REFERENCES

1 W.B. Quay, Gen. Comp. Endocrinol., 3 (1963) 473.
2 W B. Quay, Proc. Soc. Exp. Biol. Med., 115 (1964) 710.
3 D.C. Klein and J.C. Weller, In Vitro, 6 (1970) 197.
4 J. Axelrod, Science, 184 (1974) 1341.
5 D.P. Cardinari, C.A. Vagle, F Freire and J.M. Rosner, Endocrinology, 18 (1975) 72.
6 A.P. Labhsetwar, J. Endocrinol., 54 (1972) 269.
7 B. Goldmann, V. Hall, C. Hollister, P. Roychoudhury, L. Tamarkin and W. Westrom, Endocrinology, 104 (1979) 82.
8 R. J. Reiter, Endocr Rev., 1 (1980) 109.
9 E.W. Chu, R.J. Wurtman and J. Axelrod, Endocrinology, 75 (1964) 238.
10 S.Y. Ying and R.O. Greep, Endocrinology, 92 (1973) 333.
11 C.L. Ralph and H.J. Lynch, Gen. Comp. Endocrinol., 5 (1970) 334.
12 D.J. Kennaway, R.G. Frith, G. Philipou, C.D. Matthews and R.F. Seamark, Endocrinology, 101 (1977) 119.
13 M. Wilkinson, J. Arendt, J. Bradtke and D. deZeigler, J. Endocrinol., 72 (1977) 243.
14 S. Steinlechner, T.H. Champney, M.L. Houston and R.J. Reiter, Proc. Soc. Exp. Biol. Med., 175 (1984) 93.
15 F. Cattabeni, S.H. Koslow and E. Costa, Science, 178 (1972) 166.
16 B.W. Wilson, W. Snedden, R.E. Silman and P. Mullen, Anal. Biochem., 81 (1977) 283.
17 O. Beck and P. Pévet, J. Chromatogr., 311 (1984) 1.
18 I.N. Mefford and J.D. Barchas, J. Chromatogr., 181 (1980) 187.
19 G.M. Anderson, J.G. Young, D.K. Batter, S.N. Young, D.J. Cohen, and B.A. Shaywitz, J. Chromatogr., 223 (1981) 315.
20 G.M. Anderson, J.G. Young, D.J. Cohen and S.N. Young, J. Chromatogr., 228 (1982) 155.
21 H. Wakabayashi, K. Shimada and Y. Aizawa, Chem. Pharm. Bull., 33 (1985) 3875.
22 K. Imai, M. Tsukamoto and Z. Tamura, J. Chromatogr., 137 (1977) 357.
23 R.C. Causon and M.E. Carruthers, J. Chromatogr., 229 (1982) 301.
24 R.J. Martin, B.A. Bailey and R.G.H. Downer, J. Chromatogr., 278 (1983) 265.
25 S.M. Lasley, I.A. Michaelson, R.D. Greenland and P.M. Mcginnis, J. Chromatogr., 305 (1984) 27.
26 S.N. Young and G.M. Anderson, Neuroendocrinology, 35 (1982) 464.
27 S.F. Pang, F. Tang and P.L. Tang, J. Exp. Zool., 229 (1984) 41.
28 R.J. Reiter, B.A. Richardson, L.Y. Johnson, B.N. Ferguson and D.T. Dinh, Science, 210 (1980) 1372.
29 J. Axelrod and H. Weissbach, J. Biol. Chem., 236 (1961) 211.
30 R.J. Wurtman, J. Axelrod, S.H. Snyder and E.W. Chu, Endocrinology, 76 (1965) 798.