Original

Attomole Analysis of Melatonin by Precolumn Derivatization Reversed-Phase Micro-HPLC

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Abstract

An improved method for the sensitive and selective determination of melatonin was demonstrated. To improve the selectivity, extraction of melatonin derivative using ethyl acetate was adopted. MIAA (5-methoxyindole-3-acetic acid) was used as the internal standard for the precise determination. The present method was successfully applied to the determination of rat pineal melatonin (within-day precision was 1.5% and day-to-day precision was 3.2%). The sensitivity of melatonin determination using a micro-ODS column of 1.0 mm i.d. was 20 times higher than that obtained by the conventional HPLC system. Using the micro-HPLC, daytime melatonin content in C3H mouse pineal gland was determined.

Keywords: melatonin, fluorescence, micro-HPLC

Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine), a famous pineal hormone synthesized from L-tryptophan via serotonin and *N*-acetylserotonin [1], is a chemical modulator of the biological clock of vertebrates [2, 3], and clinically used against jet lag [4] and sleep disorders [5]. Melatonin is also known to have a regulatory effect on various hormones [6, 7], including gonadotropin [8], which stimulates the secretion of testosterone. In addition to these functions, because melatonin receptors are found in many organs such as the visual system [9], heart [10] and others [1, 11, 12], melatonin is thought to have yet unknown biological functions. In many cases, endogenous levels of melatonin are lower than the detectable amount of the reported methods [4, 13 - 15]. In our previous study, we reported a precolumn derivatization HPLC method for the determination of minute amount of melatonin in biological samples [16]. The detection limit of melatonin by the method is 500 amol (S / N = 5) [16], which is about 1 / 10 times lower than those of the other methods [4, 13 - 15]. The method was successfully applied to the determination of rat pineal melatonin [16, 17]. However, endogenous melatonin in other organs, such as testis and pituitary gland, could not be determined because the amount of endogenous melatonin is too small and the determination is interfered with by many endogenous substances. Therefore, a more selective and sensitive method is required. In the present investigation, an extraction procedure using various organic solvents was investigated to improve selectivity. In addition, to improve sensitivity, a micro reversed-phase HPLC system was adopted.

Experimental

Materials

Melatonin and 5-methoxyindole-3-acetic acid (MIAA) were obtained from Sigma (St. Louis, MO, USA). Methanol (MeOH), acetonitrile (MeCN) and ethyl acetate of HPLC grade were purchased from Nacalai Tesque (Kyoto, Japan). Sodium carbonate and trifluoroacetic acid (TFA) of guaranteed grade were the products of Wako (Osaka, Japan), and an aqueous solution of hydrogen peroxide (31%, v / v) was from Mitsubishi Gasukagaku (Tokyo, Japan). Water was purified by a Milli-Q system (Millipore, Bedford, MA, USA). Other reagents and solvents used were of reagent grade.

Animals

Male Wistar rats (6 weeks of age, SPF) and male C3H mice (8 weeks of age, SPF) were purchased from Seac Yoshitomi (Fukuoka,

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Japan). The animals were housed under a 12-h light and 12-h dark cycle (light on at 06:00 a.m.) and had free access to food and water.

Sample preparation

After the rat was anesthetized with diethyl ether, the pineal gland was excised quickly and homogenized at 1000 rpm 20 times in 500 μl of ice cold MeOH. After the homogenate was centrifuged at 4500g for 5 min, 50 μl of the supernatant and 50 μl of 5 nM MIAA in MeOH were mixed and the solution was dried (in the case of C3H mouse pineal gland, 200 μl of the supernatant was used and 30 μl of 1 nM MIAA was added). The calibration curve was constructed by the addition of known amounts of melatonin (25, 50 and 250 fmol) to 50 μl of the supernatant. To the residue, 40 μl of H_2O, 5 μl of aqueous 2 M Na_2CO_3 and 5 μl of aqueous 50 mM H_2O_2 (2.5 μl of 4 M Na_2CO_3 and 1 μl of aqueous 500 mM H_2O_2 were used for the mouse sample) were added and heated at 100 ˚C for 30 min. The reaction mixture was extracted 3 times with 250 μl (150 μl for mouse) of ethyl acetate. After the organic layer was dried, 50 μl (30 μl for mouse) of aqueous 10% MeCN was added to the residue, and 20 μl (5 μl for mouse) of the resulting solution was injected into the HPLC system.

Reversed-phase HPLC

A conventional reversed-phase HPLC system and a micro reversed-phase HPLC system were used for the determination of melatonin. The conventional HPLC system consisted of a PU-980 pump (Jasco, Japan), a 7725 injector (Rheodyne, Cotati, CA, USA), a CO-965 column oven (Jasco), an FP-920S fluorescence detector (Jasco) and an 807-IT integrator (Jasco). The analytical column was a TSKgel ODS-80Ts QA (150 x 4.6 mm i.d., Tosoh, Tokyo, Japan) maintained at 40 ˚C. The mobile phase was 100 mM sodium phosphate buffer (pH 7.0) containing 10% MeCN and the flow rate was 1.0 ml • min^{-1}. Fluorescence detection was carried out at 380 nm with excitation at 245 nm. For the micro-HPLC system, NANOSPACE SI-2 series (Shiseido, Tokyo, Japan) was also used. For the analytical column, CAPCELL PAK C18 MG (Shiseido) with various internal diameters (1.0, 1.5 and 2.0 mm i.d.) was used. The mobile phase was MeCN / TFA / H_2O = 10 / 0.01 / 90 (v / v). The flow rates were 0.2, 0.1 and 0.05 ml • min^{-1} for the columns of 2.0, 1.5 and 1.0 mm i.d., respectively.

Results and discussion

For the extraction of the melatonin derivative from the derivatization reaction mixture, various extraction solvents were tested. Melatonin was derivatized with hydrogen peroxide, and the derivative was extracted with several organic solvents (ethyl acetate, diethyl ether, benzene and hexane). Using diethyl ether, benzene or hexane, the melatonin derivative was hardly extracted from the reaction mixture, and more than 80% of the derivative remained in the aqueous layer. Since 86.9% of the melatonin derivative was extracted with ethyl acetate, ethyl acetate was used for the extraction. We then applied the procedure for the determination of rat pineal melatonin. Figure 1 shows the chromatograms of derivatized melatonin in rat pineal gland before and after the extraction with ethyl acetate. Before the extraction (Figure 1a), many peaks due to the endogenous substances in the pineal gland were observed. However, most of these peaks were not present in the ethyl acetate extract (Figure 1b).

In addition to developing the extraction procedure, we also investigated using an internal standard for precise determination; MIAA was selected. MIAA is derivatized with hydrogen peroxide, and the derivative shows strong fluorescence at 380 nm with excitation at 245 nm. The MIAA derivative was detected using the same conditions as for melatonin. Figure 2 shows the chromatogram of derivatized melatonin in rat pineal gland with the addition of MIAA as an internal standard. In rat pineal gland, no interfering peak was observed at the retention time of the MIAA derivative (11 min) after extraction with ethyl acetate (Figure 1b), indicating that MIAA is suitable as an internal standard for melatonin determination.

Using MIAA as an internal standard, we examined within-day and day-to-day precision of melatonin determination in rat pineal gland. Within-day precision was determined derivatizing the same sample 5 times; the obtained RSD was 1.5%. Day-to-day precision was also determined derivatizing the same sample on each day (5 days). The obtained RSD was 3.2%. Without using MIAA, these RSD values for within-day and day-to-day precision were 7.4% and 7.3%, respectively. A calibration curve of the spiked melatonin in the rat pineal gland sample was also investigated. Endogenous pineal melatonin was determined using the standard addition method. The calibration curve was linear from 10 to 100 fmol in a 20 μl injection sample with a correlation coefficient of 0.997. The endogenous melatonin content determined in rat pineal gland was 819 fmol / pineal gland (32.8 fmol / 20 μl injection), which was almost the same amount as that determined by the native fluorescence of melatonin. The melatonin content obtained in the present investigation was also consistent with the reported daytime melatonin content in rat pineal gland (500 - 1000 fmol / pineal gland) [13 - 15]. These results indicate that precise and selective determination of endogenous melatonin could be carried out by precolumn derivatization with hydrogen peroxide and extraction with ethyl acetate with the addition of MIAA as an internal standard.

To improve the sensitivity of melatonin determination, a reversed-phase micro-HPLC system was adopted. As the mobile phase, MeCN / TFA / H_2O = 10 / 0.01 / 90 (v / v) was selected, and

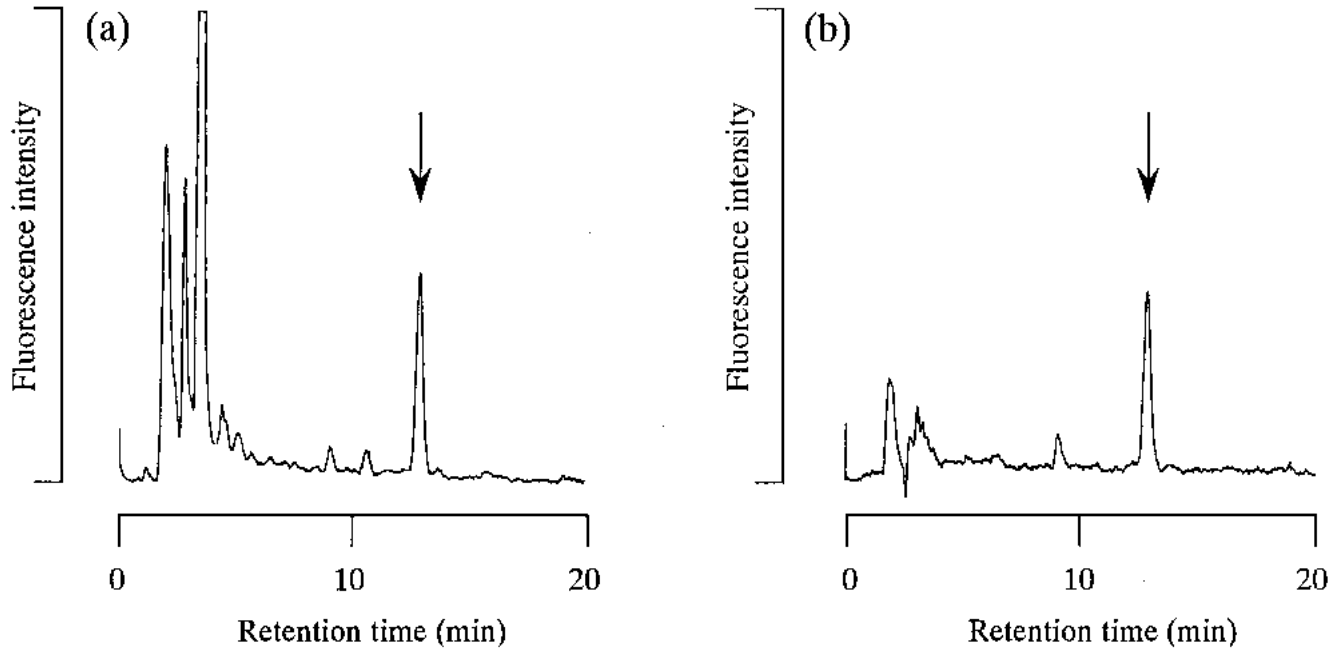

Figure 1. Chromatograms of derivatized melatonin in rat pineal gland before (a) and after (b) extraction with ethyl acetate. (a) Sample solution of rat pineal gland was derivatized with hydrogen peroxide and directly injected. (b) Derivatized melatonin was extracted from the reaction mixture and injected into the HPLC. The peak of the melatonin derivative is indicated by an arrow. HPLC conditions are described in the text.

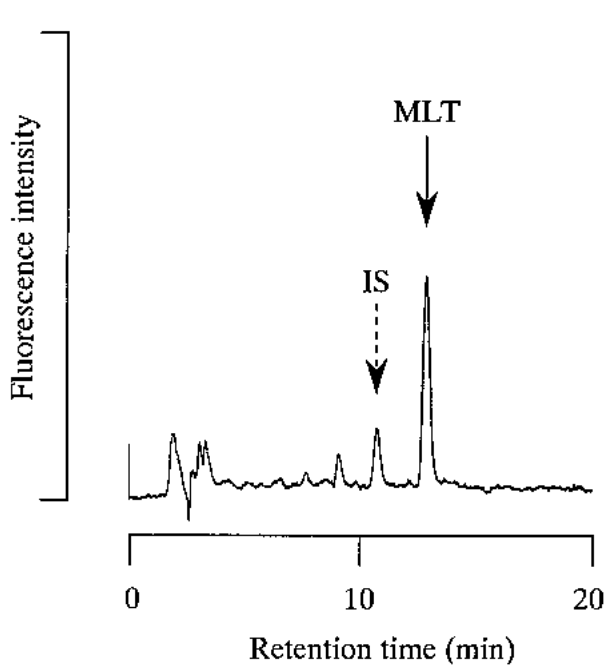

Figure 2. Chromatogram of derivatized melatonin in rat pineal gland with the addition of MIAA (IS). Peaks: IS, derivative of MIAA; MLT, derivative of melatonin. HPLC conditions are described in the text.

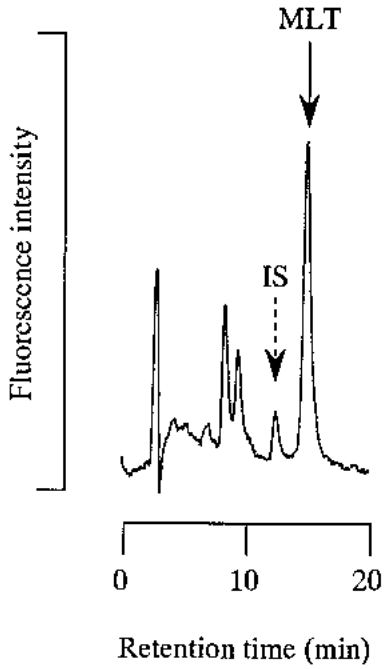

Figure 3. Chromatogram of derivatized melatonin in C3H mouse pineal gland on a micro ODS column. Peaks: IS, derivative of MIAA; MLT, derivative of melatonin. HPLC conditions are described in the text.

the derivative for 1 fmol melatonin was analyzed using a micro-ODS column with various internal diameters. The signal-to-noise ratio for the peak of the melatonin derivative became higher with a decrease of internal diameter. The highest value of 170 (S / N value for 1 fmol melatoin) was obtained with the micro-ODS column of 1.0 mm i.d.. The sensitivity of the present method was about 20 times higher than that obtained in our previous investigation [16, 17]. Using the present method, the daytime melatonin content in C3H mouse pineal gland was determined. The chromatogram is shown in Figure 3. The melatonin content was 74 fmol / pineal gland (4.9 fmol / injection); the value was consistent with that reported in our previous investigation [16]. These results indicate that attomole determination of melatonin could be carried out by the present method, which should be a powerful tool for the determination of endogenous melatonin.

References

[1] Witt-Enderby, P. A.; Li, P. -K. *Vitam. Horm*. **2000**, *58*, 321 - 354.

[2] Reppert, S. M.; Weaver, D. R.; Rivkees, S. A.; Stopa, E. G. *Science* **1988**, *242*, 78 - 81.

[3] Neu, J. M.; Niles, L. P. *Mol. Brain Res*. **1997**, *49*, 303 - 306.

[4] Sanders, D. C.; Chaturvedi, A. K.; Hordinsky, J. R. *J. Anal. Toxicol*. **1999**, *23*, 159 - 167.

[5] Jan, J. E.; Freeman, R. D.; Fast, D. K. *Dev. Med. Child Neurol*. **1999**, *41*, 491 - 500.

[6] Sainz, R. M.; Mayo, J. C.; Reiter, R. J.; Antolin, I.; Esteban, M. M.; Rodriguez, C. *FASEB J*. **1999**, *13*, 1547 - 1556.

[7] Rato, A. G.; Pedrero, J. G.; Martinez, M. A.; Rio, B. D.; Lazo, P. S.; Ramos, S. *FASEB J*. **1999**, *13*, 857 - 868.

[8] Glass, J. D.; Lynch, G. R. *Science* **1981**, *214*, 821- 823.

[9] Reppert, S. M.; Godson, C.; Mahle, C. D.; Weaver, D. R.; Slaugenhaupt, S. A.; Gusella, J. F. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 8734 - 8738.

[10] Pang, C. S.; Tang, P. L.; Song, Y.; Brown, G. M.; Pang, S. F. *Life Sci*. **1996**, *58*, 1047 - 1057.

[11] Helliwell, R. J. A.; Howell, H. E.; Lawson, W.; Barrett, P.; Morgan, P. J. *Mol. Cell. Endocrinol*. **1994**, *104*, 95 - 102.

[12] Drew, J. E.; Williams, L. M.; Hannah, L. T.; Barrett, P.; Abramovich, D. R.; Morgan, P. J. *J. Endocrinol*. **1998**, *156*, 261 - 267.

[13] Wakabayashi, H.; Shimada, K.; Aizawa, Y. *J. Chromatogr*. **1986**, *381*, 21 - 28.

[14] Vieira, R.; Miguez, J.; Lema, M.; Aldegunde, M. *Anal. Biochem*. **1992**, *205*, 300 - 305.

[15] Wurzburger, R. J.; Kawashima, K.; Miller, R. L.; Spector, S. *Life Sci*. **1976**, *18*, 867 - 877.

[16] Hamase, K.; Tomita, T.; Kiyomizu, A.; Zaitsu, K. *Anal. Biochem*. **2000**, *279*, 106 - 110.

[17] Iinuma, F.; Hamase, K.; Matsubayashi, S.; Takahashi, M.; Watanabe, M.; Zaitsu, K. *J. Chromatogr. A* **1999**, *835*, 67 - 72.