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Sensitive determination of melatonin by precolumn derivatization and reversed-phase high-performance liquid chromatography

Fumio Iinuma[b], Kenji Hamase[a], Sayaka Matsubayashi[a], Masaki Takahashi[b], Mitsuo Watanabe[b], Kiyoshi Zaitsu[a,*]

[a]*Faculty of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*
[b]*Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko-machi, Tsukui-gun, Kanagawa 199-0195, Japan*

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Abstract

A sensitive determination method for melatonin was developed. Melatonin was derivatized under alkaline conditions in the presence of hydrogen peroxide. The resultant fluorophore was excited at 247 nm and the emission wavelength was 384 nm. The Stokes shift was 137 nm, which was longer than that of melatonin itself (λ_{ex} 280 nm, λ_{em} 330 nm). The melatonin derivative was separated by reversed-phase HPLC in about 15 min and the calibration curve was linear from 500 amol to 5 pmol ($r>0.999$) with the detection limit of 500 amol ($S/N=5$). The sensitivity of this method was about ten times higher than that of previous methods. Both the day-to-day precision and within-day precision were about 5%, and the derivative of melatonin in the aqueous solution was stable for more than 10 days. This method was successfully applied to the determination of melatonin in rat pineal gland.

Keywords: Derivatization, LC; Pineal gland; Melatonin

1. Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine) is a neurohormone mainly secreted by the pineal gland [1]. It is synthesized from L-tryptophan by 5-hydroxylation, decarboxylation, *N*-acetylation and 5-methylation by the corresponding enzymes [2,3]. As the function of melatonin, the antigonadotrophic effect is well known, which suppresses the production of testosterone [4]. Recently, melatonin receptors were found in the suprachiasmatic nucleus [5], which is a pacemaker of circadian rhythm (biological clock), and melatonin was thought to modulate the rhythm of the suprachiasmatic nucleus. Furthermore, the enzymatic activities which synthesize melatonin were found in the retina [6], harderian gland [7] and testis [8] and the biological functions of melatonin in addition to its antigonadotrophic effect are of interest. Up to now, melatonin was determined by HPLC using its own fluorescence of indole compounds [9,10], HPLC–electrochemical detection (ECD) [11,12], GC [13], radioimmunoassay (RIA) [14,15] and enzyme-linked immunosorbent assay (ELISA) [16]. Although few fmol of melatonin could be determined with these methods, a more sensitive method is needed to determine the low levels of melatonin in the central nervous system and periphery. Therefore, we tried to establish a highly sensitive analytical method for melatonin in

*Corresponding author. Tel.: +81 92 6426596; fax: +81 92 6426601; e-mail: zaitsu@analysis.phar.kyushu-u.ac.jp

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biological samples. Some indole compounds such as indole, indole-3-acetic acid and tryptophan are known to give strong fluorescence under alkaline conditions in the presence of hydrogen peroxide [17,18]. We applied the reaction for the precolumn fluorescence derivatization of melatonin followed by the determination using reversed-phase HPLC, and found that the sub fmol analysis of melatonin could be achieved.

2. Experimental

2.1. Materials

Melatonin and other indole compounds were purchased from Sigma (St. Louis, MO, USA). Methanol and acetonitrile of HPLC grade were obtained from Nacalai Tesque (Kyoto, Japan). For the derivatization of melatonin, sodium carbonate and hydrogen peroxide of reagent grade were purchased from Wako (Osaka, Japan) and 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.

2.2. Fluorescence derivatization

Aqueous melatonin (30 p*M*–300 n*M*, 100 μl) was placed in glass vials and 10 μl of 300 m*M* Na_2CO_3 in H_2O and 10 μl of 10 m*M* H_2O_2 in H_2O were added. The vials were tightly capped and heated at 90°C for 30 min. The reaction mixture (20 μl) was then injected into the HPLC system.

2.3. Reversed-phase HPLC

A reversed-phase HPLC system with a fluorescence detector was used to separate and quantify the derivatized melatonin. The system consisted of a PU-980 pump (Jasco, Tokyo, Japan), a 7725 injector (Rheodyne, Cotati, CA, USA), a CO-965 column oven (Jasco), a FP-920S fluorescence detection system (Jasco) and a 807-IT integrator (Jasco). The analytical column was a J'sphere ODS-H80 (150× 4.6 mm I.D., YMC, Wilmington, NC, USA) maintained at 40°C. The eluent was 100 m*M* sodium phosphate buffer (pH 7.0) containing 12% acetonitrile, and the flow-rate was 0.5 ml/min. Fluorescence detection was carried out at 380 nm with excitation at 245 nm.

2.4. Treatment of rat pineal gland

Male Wistar rats (13 weeks of age, SPF) were anesthetized with diethyl ether and pineal gland was removed. Methanol (500 μl) was added to the sample and homogenized at 1000 r.p.m. 20 times. After centrifugation at 4500 *g* for 5 min, the supernatant (100 μl) was dried under reduced pressure, and then derivatized.

3. Results and discussion

3.1. Derivatization of melatonin

To optimize the derivatization procedure, the concentration of Na_2CO_3 and H_2O_2, reaction temperature and reaction time were examined. During each examination, the melatonin was measured using the HPLC system described in Section 2. Fig. 1 shows the effect of Na_2CO_3 concentration on the fluorescence intensity of the melatonin derivative. The peak height of melatonin becomes higher with the increase in Na_2CO_3 concentration up to 300 m*M* (the value is the concentration of derivatizing reagent; the final concentration in the reaction mixture was 25 m*M*), and gradually decreased at concentrations higher than 300 m*M*. The effect of H_2O_2 concentration was also examined. The peak height of the melatonin derivative reached a maximum when 5 m*M* H_2O_2 (final concentration was 0.42 m*M*) was used for the derivatization and did not change much when the concentration of H_2O_2 was increased. As a result, 300 m*M* Na_2CO_3 and 10 m*M* H_2O_2 were used for the derivatization of melatonin. The reaction temperature and reaction time were also investigated. The peak height of melatonin increased with the increase of reaction temperature up to 90°C, and slightly decreased at 100°C. Thus, the time course of the peak height of the melatonin derivative was examined at 90°C and 30 min was found to be the optimum time for the derivatization (Fig. 2). The excitation and emission maxima of the melatonin

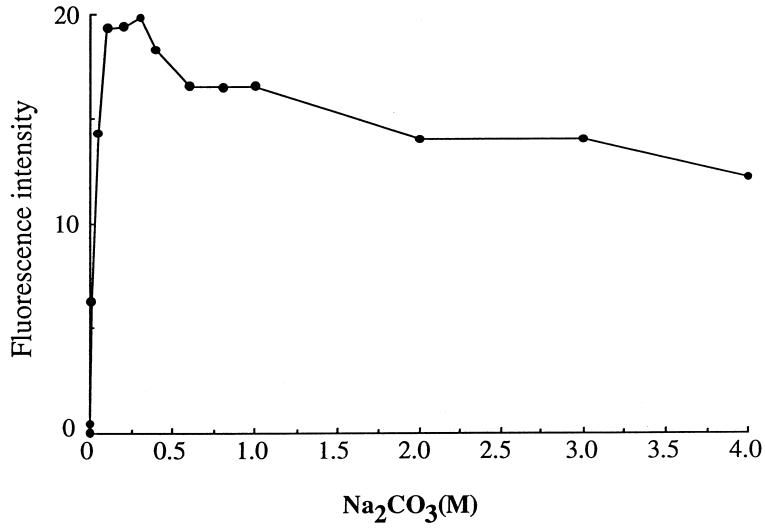

Fig. 1. Effect of sodium carbonate concentration on the fluorescence intensity for melatonin derivative. The melatonin solution (600 fmol) was mixed with Na_2CO_3 and 18 m*M* H_2O_2 then heated at 100°C for 30 min. Each plot was determined using RP-HPLC as described in Section 2 (n=3).

derivative were 247 and 384 nm, respectively (Fig. 3).

3.2. Separation of melatonin derivative

The separation conditions of the melatonin derivative for RP-HPLC were determined by considering the pH, the concentration of acetonitrile and the flow-rate of the mobile phase. Neither the peak height of the melatonin derivative nor the retention time changed on changing the pH of the mobile phase from 5.0 to 8.0. The concentration of acetonitrile and the flow-rate of the mobile phase were examined by the elution of tryptophan, 5-hydroxytryptophan, serotonin, *N*-acetylserotonin, 5-methoxy-

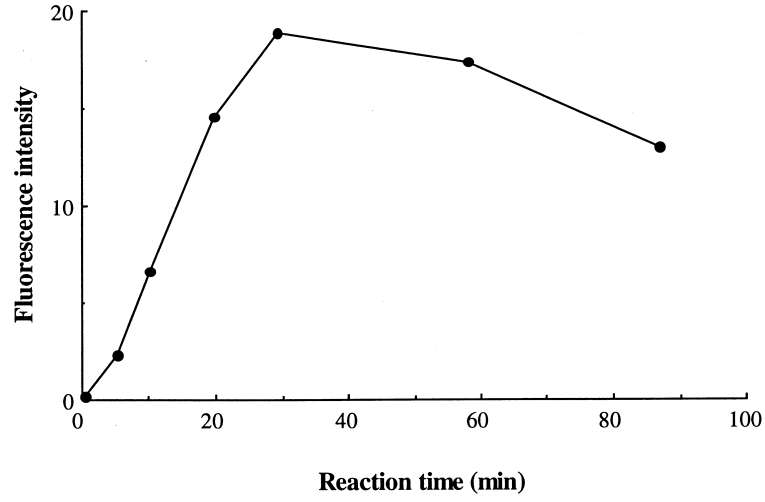

Fig. 2. Effect of reaction time on the fluorescence intensity for melatonin derivative. The melatonin solution (600 fmol) was mixed with 300 m*M* Na_2CO_3 and 10 m*M* H_2O_2 then heated at 90°C. Each plot was determined using RP-HPLC as described in Section 2 (n=3).

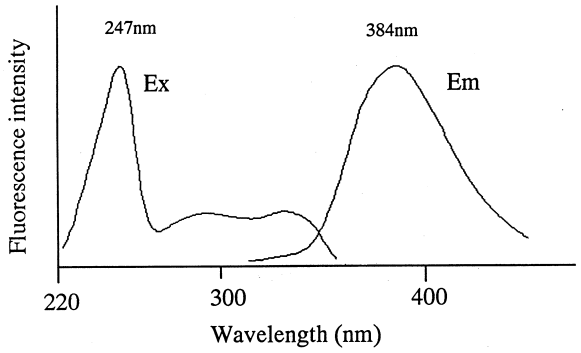

Fig. 3. Fluorescence excitation and emission spectra of melatonin derivative. Excitation spectrum was measured with emission at 384 nm, emission spectrum was measured with excitation at 247 nm.

tryptophan, 5-methoxyindoleacetic acid and melatonin, which are considered to be the major components of the indole compounds in the pineal gland. These compounds were derivatized by the same procedure as melatonin and separated by RP-HPLC. Using the mobile phase containing >14% acetonitrile, 5-methoxyindoleacetic acid was not separated from the melatonin derivative. A lower acetonitrile content (<10%) or a lower flow-rate (<0.5 ml/min) resulted in a time consuming procedure, therefore, 100 m*M* sodium phosphate buffer (pH 7.0) containing 12% acetonitrile at a flow-rate of 0.5 ml/min was selected for the mobile phase.

3.3. Calibration curve and detection limit

The melatonin solutions (30 p*M*–300 n*M*) were derivatized and the linearity of the calibration curve was studied (500 amol–5 pmol/20 μl inject). The calibration curve was linear from 500 amol to 5 pmol (20 μl loop injection) with the correlation coefficient was higher than 0.999. The detection limit of the melatonin was 500 amol ($S/N=5$). Since the detection limits of the previous methods [9–16] were of the order of few fmols, the sensitivity of this method was about ten times higher than that of the previous methods. The chromatograms for 50 fmol melatonin using the present method and using the native fluorescence of melatonin are shown in Fig. 4.

3.4. Validation of the method and stability of the derivative

Repeatability and day-to-day precision of the method were examined using 50 fmol melatonin. Repeatability was determined by analyzing eight

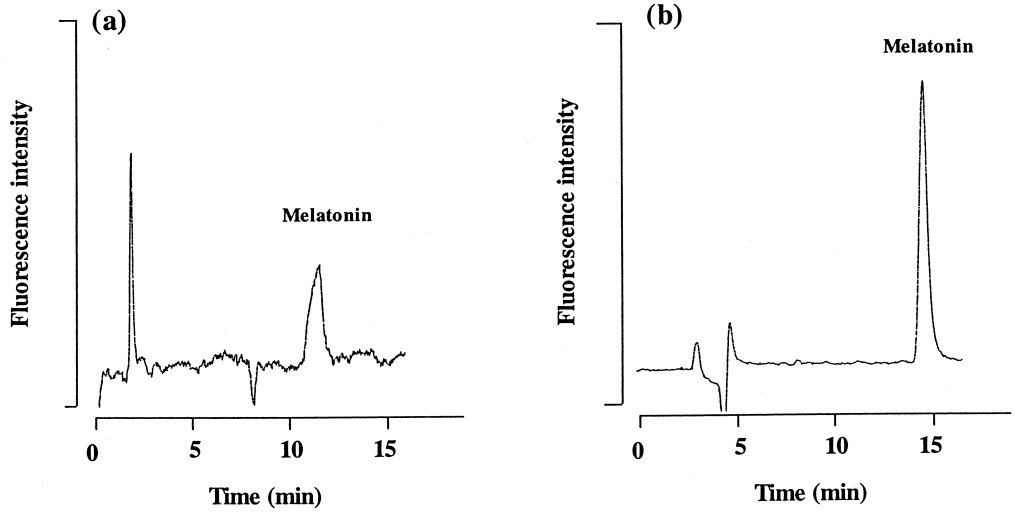

Fig. 4. Chromatograms of (a) 50 fmol melatonin and (b) derivative for 50 fmol melatonin. The HPLC conditions of (a) were as follows. column: ODS-80T$_M$ (150×4.6 mm I.D., Tosoh, Tokyo, Japan) maintained at 40°C, mobile phase: 10 m*M* NaOAc 2 μ*M* EDTA (pH 4.0)–MeOH (70:30, v/v), flow-rate: 1.0 ml/min, detection: fluorescence (λ_{ex} 280 nm, λ_{em} 330 nm). HPLC conditions of (b) as described in the text.

samples within a day and the obtained RSD was 5.8 %. The day-to-day precision was determined by analyzing 50 fmol of melatonin for 5 days. The data obtained on each day was the average of two analyses. The obtained RSD was 4.0%. The stability of the melatonin derivative was also investigated. The derivatized melatonin samples were stored at 4°C and analyzed using RP-HPLC for 10 days. The melatonin derivative was stable for at least 10 days at 4°C in the dark.

3.5. Determination of melatonin in rat pineal gland

Pineal gland is an important tissue which synthesizes and releases melatonin. Therefore, we applied the present method for the determination of melatonin in rat pineal gland. Because the melatonin content was the lowest at daytime, we applied our present method to the determination of daytime melatonin content in rat pineal gland. The derivatizing reaction conditions for the rat pineal melatonin was examined by using standard addition method considering the fluorescence intensity (peak area) of the melatonin derivative. As a result, 2 *M* Na_2CO_3 and 50 m*M* H_2O_2 were selected and the reaction was carried out at 100°C for 30 min. Melatonin content obtained by the present method was 850 fmol/pineal gland. The chromatogram is shown in Fig. 5. Pineal melatonin of the same sample was also determined using the native fluorescence of indoles according to the method described in Fig. 4a. The melatonin content was 870 fmol/pineal gland, which was almost the same amount as that obtained by the present method. The result obtained in this paper was consistent with the reported daytime melatonin content in rat pineal gland of 500–1000 fmol [12,19].

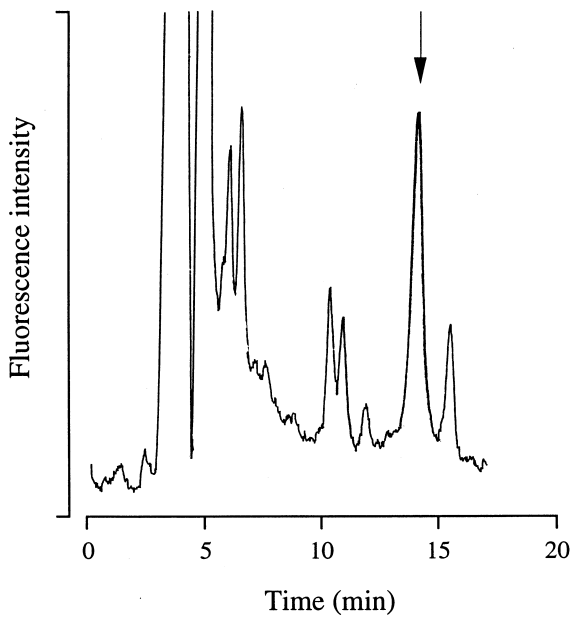

Fig. 5. Determination of melatonin in rat pineal gland. The peak for melatonin is indicated by an arrow. Derivatization and HPLC conditions are described in the text.

4. Conclusion

A sensitive method for the analysis of melatonin using a novel precolumn fluorescence derivatization and RP-HPLC was demonstrated. With this method, subfemto mol analysis of melatonin could be accomplished, which is the most sensitive to date, and this method was successfully applied to the accurate analysis of rat pineal melatonin. Good repeatability and day-to-day precision was obtained and the derivative was stable for more than 10 days. Based on the results described in this paper, this method appears promising for the determination of minute amounts of melatonin, and may be a powerful tool for the analysis of various biological samples.

References

[1] L. Tamarkin, C.J. Baird, O.F.X. Almeida, Science 227 (1985) 714.
[2] J. Axelrod, Science 184 (1974) 1341.
[3] D. Sugden, Experiencia 45 (1989) 922.
[4] F.L. Jackson, J.J. Heindel, J.P. Preslock, A.S. Berkowitz, Biol. Reprod. 31 (1982) 436.
[5] M.L. Dubocovich, S. Benloucif, M.I. Masana, Behav. Brain Res. 73 (1996) 141.
[6] A.F. Wiechmann, K. O'Steen, Brain Res. 506 (1990) 14.
[7] A. Menendez-Pelaez, K.A. Howes, A. Gonzales-Brito, R.J. Reiter, Biochem. Biophys Res. Commun. 145 (1987) 1231.
[8] M. Tijmes, R. Pedraza, L. Valladares, Steroids 61 (1996) 65.
[9] M.H. Mills, M. King, J. Chromatogr. 377 (1986) 350.
[10] H. Wakabayashi, K. Shimada, Y. Aizawa, J. Chromatogr. 381 (1986) 21.

[11] Y. Sagara, Y. Okatani, S. Yamanaka, J. Chromatogr. 431 (1988) 170.
[12] R. Vieira, J. Miguez, M. Lema, Anal. Biochem. 205 (1992) 300.
[13] C.R. Lee, H. Esnaud, Biomed. Environ. Mass Spectrom. 15 (1988) 249.
[14] R.J. Wurzburger, K. Kawashima, R.L. Miller, Life Sci. 18 (1976) 867.
[15] A. Miles, D. Phibrick, S.F. Tidmarsh, Clin. Chem. 31 (1985) 1412.
[16] S-M. Yie, E. Johansson, G.M. Brown, Clin. Chem. 39 (1993) 2322.
[17] F. Iinuma, K. Mawatari, M. Tabara, M. Watanabe, J. Pharm. Dym. 7 (1984) S-10.
[18] G-N. Chen, X-Q. Xu, J-P. Duan, M-M. He, F. Zhang, Analyst 120 (1995) 1699.
[19] G.M. Anderson, J.G. Young, D.J. Cohen, J. Chromatogr. 228 (1982) 155.