JOURNAL OF CHROMATOGRAPHY. B, BIOMEDICAL AND APPLICATIONS (2000) 747(1-2):95-110.

Separation and assay methods for melatonin and its precursors

Harumi T, Matsushima S

Review article

Separation and Assay Methods for Melatonin and Its Precursors

Tatsuo Harumi* and Shoji Matsushima

Department of Anatomy, Asahikawa Medical College, Asahikawa 078-8510, Japan

*Corresponding author.

TEL: +81-166-68-2312

FAX: +81-166-68-2319

e-mail: harumi@asahikawa-med.ac.jp

Abstract

Melatonin is an indoleamine hormone that is synthesized from tryptophan via 5-hydroxytryptophan, serotonin and N-acetylserotonin in the vertebrate pineal gland. Many chromatographic and non-chromatographic techniques have been developed and improved for the determination and measurement of melatonin and its related indoleamines. At present, gas chromatography with mass spectrometry and reversed-phase high performance liquid chromatography with fluorescence or electrochemical detection are widely used for indoleamine determinations in the pineal gland. This review will deal with methods for the separation and determination of the melatonin and its related indoleamines.

Keywords: Melatonin; Indoleamine; Pineal gland; GC/MS; HPLC

Contents

1. Introduction

Melatonin is an indoleamine hormone derived from tryptophan and it mediates many physiological, endocrinological and behavioral processes including the regulation of circadian rhythm, sleep, mood, reproduction, immune response and aging in the vertebrate body [1-3]. Melatonin was first isolated from the pineal gland as a potent frog skin-lightening agent, and was characterized as N-acetyl-5-methoxytryptamine in 1959 by Lerner *et al.* [5,6]. After the characterization of melatonin, many researchers developed new methods, and improved the existing methods to determine melatonin in the pineal gland, blood plasma and other organs with biological and biochemical techniques. In addition, since the contents of melatonin, its precursors and its related indoleamines in the pineal gland of most mammalians show clear circadian changes, quantitative analyses of the pineal indoleamines have also been performed at the same time as melatonin analysis. To determine the amount of each indoleamine, methods for separation including several chromatographic techniques have been developed and improved. At present, it is known that melatonin is present even in invertebrates [7] and plants [8]. This review

will summarize how melatonin and its related indoleamines have been analyzed qualitatively and quantitatively.

2. Melatonin biosynthesis and indoleamine metabolism in the pineal gland

The pathway of melatonin synthesis and indoleamine metabolism in the pineal gland is illustrated in Fig. 1. The parenchyma of the mammalian pineal gland is predominantly composed of a group of cells called pinealocytes. Pinealocytes take up tryptophan (Trp) from the circulation. Although the transport system of the Trp into the pinealocytes has not been made clear yet, the incorporated Trp is hydroxylated to 5-hydroxytryptophan (HTrp) by tryptophan hydroxylase. Subsequently, HTrp is decarboxylated to 5-hydroxytryptamine (5HT: serotonin) through the action of aromatic-L-amino acid decarboxylase. Serotonin-N-acetyltransferase (SNAT), which regulates the rate of melatonin biosynthesis in the pineal gland, catalyzes the acetylation of 5HT to N-acetylserotonin (NAS). Finally, a methyl group from S-adenosylmethionine is transferred to NAS by hydroxyindole-O-methyltransferase (HIOMT), and NAS is converted to 5-methoxy-N-acetyltryptamine, or melatonin (Mel).

The resulting Mel is highly nonpolar because the potentially charged 5-hydroxy and amine groups in 5HT are blocked by the action of SNAT and HIOMT. One of the alternative metabolic pathways of 5HT is its oxidative deamination to 5-hydroxyindole acetaldehyde catalyzed by monoamine oxydase (MAO), and is followed by the rapid conversion to 5-hydroxyindole acetic acid (HIAA) and 5-hydroxytryptophol (HTPL). Another pathway is methylation of 5HT to 5-methoxytryptamine (5MT) catalyzed by HIOMT. The HIOMT also catalyzes the methylation of HTrp, HIAA and HTPL to 5-methoxytryptophan (MTrp), 5-methoxyindole acetic acid (MIAA) and 5-methoxytryptophol (MTPL), respectively. In most mammalian species, the contents of Mel and its precursor, NAS, in the pineal gland show clear circadian changes with the highest level occurring during the dark period. This elevation of Mel and NAS amounts observed in the dark period is due to the elevation of SNAT activity and the expression of the SNAT gene [9]. On the other hand, the 5HT amount in the pineal gland shows circadian changes opposite to the Mel and NAS rhythm. These circadian changes of 5HT amount in the pineal gland may relate to its cyclic production from Trp, metabolism to NAS, and release from the pinealocytes. Not only 5HT, but also HIAA, MIAA, HTPL and MTPL show circadian changes parallel with the

5HT rhythm [3,39].

3. Bioassay of melatonin

Bioassay using tadpole skin was first reported as a specific and quantitative determination method for Mel [10]. This assay utilizes the aggregation of the melanin granules within the living dermal melanophore induced by Mel (Fig. 2A). By assessing the dermal melanophore responded to Mel in terms of the five-stage Hogben melanophore index, a linear relationship was observed between the log of melatonin concentration from 0.4 to 4.3 pmol/l (0.1 to 1 ng/ml) and the melanophore index (Fig. 2B).

4. Separation and determination methods of melatonin and its related indoleamines

4.1. Planer techniques

Lerner *et al.* first demonstrated that Mel is N-acetyl-5-methoxytryptamine [5,6]. The Mel purified from bovine pineal and synthetic N-acetyl-5-methoxytryptamine were identical in their properties, such as fluorescence, ultraviolet absorption, elution profile from silicic acid and aluminum oxide columns, countercurrent distribution and bioassay. Furthermore, paper chromatography using six different solvent systems also indicated that all *Rf* values of the Mel from bovine pineal gland and synthetic N-acetyl-5-methoxytryptamine were identical. Separation of indoleamines by paper chromatography was described by Kveder and McIsaac [11]. Ten indoleamines including Mel, NAS, 5HT, HIAA, MIAA and 5MT on Whatman No. 1 paper were developed with *n*-propanol-ammonia (8:2, v/v) or *n*-butanol-acetic acid-water (4:1:5, v/v). Each indoleamine was detected by the fluorescence under ultraviolet lamp or by colored spot with six different dyes. Klein and Notides employed a two-dimensional thin-layer chromatography for the separation of Mel, NAS, HTPL, HIAA, MIAA and MTPL [12]. A silica gel coated plate was first developed with chloroform-methanol-glacial acetic acid (93:7:1, v/v). In the second direction, the plate was developed with ethyl acetate. For fluorescent visualization of the indoleamines, the plate was sprayed with methanol/12.5 M HCl, and irradiated by

ultraviolet light. In this solvent system, Trp, HTrp, 5HT and 5MT stayed at the origin (Fig.3).

4.2. Liquid-phase extraction

Before column chromatographic techniques were applied for indoleamine separations, liquid-phase extraction was examined for the separation of indoleamines [13,14]. As shown in Fig. 4, the liquid-phase extraction method resulted in high recovery of 5HT, 5MT and Mel. In addition, both of the two papers introduced sensitive fluorometric determination methods for indoleamines. Quay [13] determined the specific fluorescence of 5-hydroxy- and 5-methoxyindoleamines at 540-550 nm when excited at 295 nm in 3N HCl. Miller and Maickel reported that the indoleamines yielded highly fluorescent compounds at 470 nm when excited at 360 nm after the indoleamines reacted with *o*-phthalaldehyde [14]. Using this technique, the amounts of 5HT, NAS, Mel and 5MT in the pineal gland of dog and rat were measured [14].

4.3. Gas chromatography and gas chromatography-mass spectrometry

Gas chromatography coupled with an argon ionization detector [15-17] or a flame ionization detector [18,19] was applied for the separation and determination of authentic indoleamines. In these studies, intact indoleamines [15,17,18], acetylated derivatives [16], and trimethylsilyl and trifluoroacetyl derivatives (Fig. 5) [19] of the indoleamines were analyzed. Cole and Crank favored the use of non-polar liquid phase, such as SE-30, and silyl derivatization of indoleamines to lower the column temperatures [19]. Later, the application of gas chromatography-mass spectrometry (GC/MS) led to the quantitative analysis of Mel [20-26], 5HT [21-23, 27], MTPL [24-26], 5MT [21,22,27], and MIAA [26] in the pineal gland [20-22,24-26] and hypothalamus [21,22,27]. Appropriate derivatization of Mel and other indoleamines gives them adequate vapor pressure for gas chromatography. Derivatization of authentic Mel and biogenic samples were performed with a silanizing agent to form trimethylsilyl melatonin (TMS-Mel) [24,25], with heptafluorobutyrylimidazole to form diheptafluorobutyryl melatonin (HFB-Mel) [20], or with pentafluoropropionic anhydride to form pentafluoropropionyl melatonin (PFP-Mel) [21-23,26,27]. Fig. 6 shows mass spectra of Mel and Mel derivatives in electron impact mass spectrometry. TMS-Mel

gives rise to two major fragment ions at m/z 232 and 245 due to the and cleavages of side chains to the pyrrole ring (Fig. 6B). Since the fragment ions at m/z 232 and 245 derived from TMS-Mel are much higher than the molecular ion at m/z 304, a standard curve for quantitative analysis was made with the ratio of the peak height of the fragment ion at m/z 232 to the peak height of the internal standard [24,25]. The peak height of fragment ions of HFB-Mel appeared at m/z 159, 356 and 369 are also higher than the height of the molecular ion at m/z 582 (Fig. 6C). On the other hand, PFP-Mel is a spirocyclic derivative [28,29]. The molecular ion of this derivative at m/z 360 is the most abundant ion on the mass spectrum, and its fragment ions at m/z 186 and 213 appear as smaller peaks (Fig. 6D). Therefore, the peak height of the molecular ion at m/z 360 is utilized for the quantitative analysis using PFP-Mel [21,22,26,27]. While PFP-Mel contains one pentafluoropropionyl (PFP) group, the PFP derivatives of 5HT and 5MT contain three PFP groups and two PFP groups, respectively [21]. Jamieson and Hutzinger analyzed the mass spectra of eight synthetic indoleamines including Mel, NAS, HIAA, MIAA, HTPL and MTPL without derivatization by direct introduction [30]. All the eight indoleamines yielded a single molecular ion and abundant fragment ions due to the and cleavages of

the side chain to the pyrrole ring. On the mass spectrum of Mel, fragment ions at m/z 160 and 173 are abundant (Fig. 6A). The former ion (m/z 160) is also formed from MIAA and MTPL [30]. All analyses described above were performed with electron impact mass spectrometry.

4.4. High performance liquid chromatography

High performance liquid chromatography (HPLC) with an isoclatic mobile phase is a widely used technique for the qualitative and quantitative analyses of biogenic amines. Since the HPLC system allows high resolution and sensitive detection of indoleamines with a relatively simple method for sample preparation, many investigators have applied the system for separation of pineal Mel and indoleamines [31-44]. Vitale *et al.* reported that the HPLC assay using a normal-phase column and an organic non-polar mobile phase is a sensitive method for the Mel determination [31]. However, almost all other investigators used HPLC using reversed-phase columns with an aqueous mobile phase for the determination of Mel (Table 1).

Reversed-phase HPLC (RP-HPLC) is useful for separation of multiple

indoleamines on one chromatogram. However, since pineal indoleamines contain a wide range of polar and non-polar compounds, more than one HPLC mobile phases were used to resolve the pineal indoleamines. Anderson *et al.* reported the use of HPLC mobile phases containing 12% methanol for Trp, 5HT, HIAA and NAS, and 35% methanol for Mel determinations [32,33]. Mills *et al.* reported the use of a mobile phase containing 6.8% acetonitrile for Trp, HTrp, MTrp, 5HT, HIAA, HTPL and NAS, and 16% acetonitrile for Mel determinations (Fig. 7) [37]. Mefford and Barchas used the mobile phase without methanol for Trp, 5HT, HIAA and HTPL, with 10% methanol for MTrp and NAS, and 25% methanol for Mel determinations [38]. Furthermore, Mefford *et al.* used the mobile phase containing 10% acetonitrile for Trp and hydroxyindoles, and 20% acetonitrile for methoxyindoles and NAS determinations (Fig. 8) [39].

When a polar or ionized solute is mixed with an ion holding opposite electrical charge or a counter ion, an ion-pair is formed between the solute ion and the counter ion. The resultant complex has a lower net electrical charge or polarity. Since polarity or hydrophobicity of samples is a major factor determining the retention time on RP-HPLC, addition of counter ions or ion paring agents into the RP-HPLC mobile

phase results in a change of the retention time of the polar samples. Many kinds of ion paring agents including alkylammonium ions, alkylsulphonates, inorganic ions and surface-active ions were often utilized for the separation of polar samples by RP-HPLC [45]. Raynaud and P vet used triethylamine (TEA) and sodium 1-octanesulfonate (SOS) as ion pairing agents for the determination of 5MT, MIAA, MTPL and Mel [44]. Addition of TEA (0.1%) into the RP-HPLC mobile phase resulted in an increase in the retention time of MIAA, but a slight decrease in the retention time of 5MT. In contrast, addition of SOS (0.2 mm) increased the retention time of 5MT, but slightly decreased the retention time of MIAA. Retention times of both Mel and MTPL were not affected with the existence of the ion paring agents. For the simultaneous determination of Mel and its precursors, 5HT and NAS, addition of alkylsulphonate ion-paring agents to the HPLC mobile phase was examined [36,41]. Chin reported the simultaneous determination of Trp, HTrp, 5HT, NAS, Mel and HIAA by RP-HPLC with 4 mm sodium octylsulfonate [36]. The effect of SOS concentrations in the HPLC mobile phase on the retention time was examined by Harumi *et al.* [41]. As shown in Fig. 9, the retention time of 5HT is increased as the SOS concentration is raised. On the other hand, increasing the SOS concentration from 0 to 3 mm

results in a slight decrease of the retention time of MTrp, MTPL and Mel. The retention time of other indoleamines, Trp, NAS, HIAA and MIAA were almost unaffected by the SOS concentrations tested (0-5 mm). Fig. 10 shows typical chromatograms of the standards for 5HT, NAS and Mel, and an extract from a single pineal gland of a golden hamster. At present, there is no report of the use of other ion paring agents, such as sodium dodecyl sulfate, for the HPLC analyses of pineal indoleamines.

After the chromatographic separation of pineal indoleamines, either or both kinds of detection methods were used. One is fluorometric detection (FD) (Fig. 7) [31-37], and the other is electrochemical detection (ECD) (Fig. 8) [32,33,36,38-44]. In the FD of pineal indoleamines, excitation and emission wavelengths are set at 285 and 345 nm [31-34], 297 and 341 nm [35], 232 and 353 nm [36], 280 and 340 nm (Fig. 7) [37], and 286 and 352 nm [38], respectively. In the ECD of indoleamines, the setting of the applied potential is important. Usually, an applied potential greater than 800 mV results in a higher sensitivity of Mel detection (Fig. 11A). However, a higher potential causes an elevation of the baseline noise (Fig. 11B). The applied potential for the Mel determination is set at 700 mV [32,33], 850 mV [36], 900 mV (Fig. 8, 10)

[38-43] and 1.25 V [44]. A dual ECD system is favored for the determination of methoxyindoles [44]. As shown in Fig. 11A, hydroxyindoles, such as 5HT and NAS, are more readily oxidized than methoxyindoles, such as Mel. Therefore, when the first detector is maintained at a lower potential (350 mV) than the second (1.25 V), hydroxyindoles are totally oxidized at the first detector, and 5MT, MIAA, MTPL and Mel are specifically detected in the second detector [44].

5. Determination of melatonin in blood plasma

Melatonin is secreted from the pineal gland into the blood, and works as a circulating hormone. Reflecting the nocturnal increase of Mel synthesis in the pineal gland, plasma Mel level also increases in the dark period. Since normal daytime plasma melatonin levels are extremely low (< 43 pmol/l; 10 pg/ml), a specific and sensitive assay method was taken. Radioimmunoassay (RIA) is widely used for the measurement of plasma Mel levels because the sensitivity is high, large numbers of samples can be measured at a time, and several commercial kits are available [46]. On the other hand, GC/MS [47-54] and RP-HPLC with either FD [55-57] or ECD (Fig.

12) [42-44,58] are also useful for the Mel analysis in the blood plasma.

In the early works of the plasma Mel analysis by GC/MS, Mel and plasma were derivatized by silanization [48,49]. Later, sample derivatization was performed with pentafluoropropionic anhydride to form PFP-Mel [50-54]. In addition, the use of deuterium-labeled Mel or N-acetyl-5-methoxytryptamine as an internal standard improved the sensitivity and reliability of the Mel assay by GC/MS [50-54]. Markey [52] and Skene *et al.* [53] reported that negative ion chemical ionization (CI) mass spectrometry is useful for the detection of less than 4.3 pmol/l (1 pg/ml) of Mel, and is more sensitive than the electron impact (EI) mass spectrometry. However, Lee and Esnaud reported later that EI mass spectrometry with a capillary GC gives adequate sensitivity for blood Mel analysis [54].

In the RP-HPLC analysis for Mel in the pineal gland, extraction of the Mel from the pineal gland is carried out using an easy step, such as acid extraction with 10% perchloric acid. However, for the determination of Mel in blood plasma, several prior extraction steps are required for sample preparation to eliminate non-specific interference. The prior extraction of Mel from plasma is performed with a liquid phase using organic solvents, such as dichloromethane [42-44] and chloroform [58], or by

solid phase extraction (SPE) with C_{18} octadecylsilyl columns [55,57]. On the other hand, Bechgaard *et al.* reported that RP-HPLC with FD of plasma without prior extraction could be useful for bioavailability studies of Mel after intravenous and intranasal administration [56].

The prior extraction step is also required for sample preparation in the GC/MS analysis of plasma Mel. Lee and Esnaud reported the use of an SPE column containing normal phase silica for the prior extraction [54]. However, they also warned that the recovery rate of Mel from prepacked SPE column varies among the batches and manufacture of the columns, and was very poor in some columns [59].

*6. Conclusion*s

At the present time, the quantitative analyses of both Mel and its related indoleamines in the pineal gland are mostly performed by two methods, GC/MS and HPLC. Although GC/MS achieves highly sensitive and quantitative analyses of indoleamines, the cost of its apparatus and maintenance is more expensive than HPLC. In addition, appropriate sample derivatization, such as PFP derivatization, and

deuterium-labeled internal standards are required for the accurate quantitative determination of indoleamines by GC/MS. Therefore, in the routine analyses of the indoleamines, HPLC with either FD or ECD seem to be employed more easily when compared with GC/MS. Which is the better detection method for pineal indoleamine analysis, FD or ECD? Anderson *et al.* reported that the detection limit of Mel by FD is 25 pg while the limit by ECD is 50 pg [32]. Chin also reported that the detection limit of Mel by FD is 60 pg, and the limit by ECD is 135 pg [36]. Iinuma *et al.* reported that the precolumn derivatization of Mel under alkaline conditions in the presence of hydrogen peroxide resulted in a detection limit of 116 fg (500 amol) by FD [60]. On the other hand, the detection limit by ECD is reported as 20 pg [38], 8 pg [43], 5 pg [38,41] and 4 pg [44] without derivatization, whereas the limit by FD was reported as 35 pg [35] and 10 pg [37]. Therefore, in the routine Mel analyses without derivatization, ECD seems to be the more sensitive method compared with FD. One disadvantage of ECD is that a higher concentration of organic solvent, such as methanol or acetonitrile, in the HPLC mobile phase shortens the life time of the working electrode in the detector. Therefore, the concentration of organic solvent in the mobile phase for ECD is usually held at less than 30% (v/v). On the other hand,

in the FD, there is no limit for the concentration of organic solvents, and FD shortens the retention time of Mel by increasing the methanol or acetonitrile concentration in the HPLC mobile phase. In relation to this, Kulczykowska and Iuvone reported the case of plasma Mel analysis by HPLC with FD using the mobile phase containing 60% methanol [57]. In addition, ECD is less sensitive for the Trp molecule than FD [32]. An additional advantage of FD is that the materials detected by FD could be retrieved after detection. Since ECD is a destructive examination, the detected materials by ECD decomposed. Hence, HPLC with either FD and ECD has better points. For the separation and determination of pineal indoleamines, whereas RP-HPLC and GC/MS have been widely used, other techniques, such as high performance thin-layer chromatography, liquid chromatography-mass spectrometry, micellar electrokinetic chromatography and capillary electrophoresis (CE), will also be applicable. Although the CE technique was applied for the analyses of 5HT level in brain microdialysates [61], amount of 5HT release from mast cells [62] and authentic hydroxyindoles [63], at present, successful determination of pineal indoleamines including Mel by these techniques has not been achieved yet.

7. Acknowledgments

We would like to thank Mr. H. Akutsu (Central Laboratory for Research and Education, Asahikawa Med. College) for technical assistant in GC/MS analysis, and Dr. N. Suzuki (Hokkaido Univ.) for critical reading of this manuscript. We would also thank Drs. D. C. Klein, S. P. Markey and H. Y. Kim (NIH) for providing us with valuable information and suggestions. Our work in the field of research on the pineal gland is supported, in part, by Grants-in-Aid 11740452 from the Ministry of Education, Science and Culture of Japan and a grant from The Akiyama Foundation.

References

[1] P.B. Foley, K.D. Cairncross and A. Foldes, Neurosci. Biobehavi. Rev., 10 (1986) 273.

[2] D. Sugden, Experientia, 45 (1989) 922.

[3] R.J. Reiter, Endocrine Rev., 12 (1991) 151.

[4] A. Brzezinski, N. Engl. J. Med., 336 (1997) 186.

[5] A.B. Lerner and J.D. Case, J. Am. Chem., 81 (1959) 6084.

[6] A.B. Lerner, J.D. Case and Y. Takahashi, J. Biol. Chem., 235 (1960) 1992.

[7] B. Vivien-Roels and P. P vet, Experientia, 49 (1993) 642.

[8] A. Hattori, H. Migitaka, M. Iigo, M. Itoh, K. Yamamoto, R. Ohtani-Kaneko, M. Hara, T. Suzuki and R. J. Reiter, Biochem. Mol. Biol. Int., 35 (1995) 627.

[9] D.C. Klein, S.L. Coon, P.H. Roseboom, J.L. Weller, M. Bernard, J.A. Gastel, M. Zatz, P.M. Iuvone, I.R. Rodriguez, V. Begay, J. Falcon, G.M. Cahill, V.M. Cassone and R. Baler, Recent Prog. Horm. Res., 52 (1997) 307.

[10] C.L. Ralph and H.J. Lynch, Gen. Comp. Endocrinol., 15 (1970) 334.

[11] S. Kveder and W.M. McIsaac, J. Biol. Chem., 236 (1961) 3214.

[12] D.C. Klein and A. Notides, Anal. Biochem., 31 (1969) 480.

[13] W.B. Quay, Anal. Biochem., 5 (1963) 51.

[14] F.P. Miller and R.P. Maickel, Life Sci., 9 (1970) 747.

[15] H.M. Fales and J.J. Pisano, Anal. Biochem., 3 (1962) 337.

[16] C.J.W. Brooks and E.C. Horning, Anal. Chem., 36 (1964) 1540.

[17] M. Greer and C.M. Williams, Clin. Chim. Acta, 15 (1967) 165.

[18] S.N. Pennington, J. Chromatogr., 32 (1968) 406.

[19] E.R. Cole and G. Crank, J. Chromatogr., 61 (1971) 225.

[20] P.H. Degen, J.R. DoAmaral and J.D. Barchas, Anal. Biochem., 45 (1972) 634.

[21] F. Cattabeni, S.H. Koslow and E. Costa, Science, 178 (1972) 166.

[22] S.H. Koslow and A.R. Green, Adv. Biochem. Psycopharm., 7 (1973) 33.

[23] S.H.Koslow, Adv. Biochem. Psychopharm., 11 (1974) 95.

[24] B.W. Wilson, H.J. Lynch and Y. Ozaki, Life Sci., 23 (1978) 1019.

[25] B.W. Wilson, J. Neural Transm. Suppl., 13 (1978) 279.

[26] O. Beck and P P vet, J. Chromatogr., 311 (1984) 1.

[27] A.R. Green, S.H. Koslow and E. Costa, Brain Res., 51 (1973) 371.

[28] K. Blau, G.S. King and M. Sandler, Biomed. Mass Spectrom., 4 (1977) 232.

[29] K.M. Biswas, A.H. Jackson and M. Tehrani, J. Chem. Soc. Chem. Commun., (1982) 765.

[30] W.D. Jamieson and O. Hutzinger, Anal. Biochem., 37 (1970) 182.

[31] A.A. Vitale, C.C. Ferrari, H. Aldana and J.M. Affanni, J. Chromatogr. B, 681 (1996) 381.

[32] G.M. Anderson, J.G. Young and D.K. Batter, J. Chromatogr., 223 (1981) 315.

[33] G.M. Anderson, J.G. Young, D.J. Cohen and S.N. Young, J. Chromatogr., 228 (1982) 155.

[34] H. Wakabayashi, K. Shimada and Y. Aizawa, J. Chromatogr., 381 (1986) 21.

[35] J.L. Chin, J. Chromatogr., 428 (1988) 206.

[36] J.R.L. Chin, J. Chromatogr., 528 (1990) 111.

[37] M.H. Mills, D.C. Finlay and P.R. Haddad, J. Chromatogr., 564 (1991) 93.

[38] I.N. Mefford and J.D. Barchas, J. Chromatogr., 181 (1980) 187.

[39] I.N. Mefford, P. Chang, D.C. Klein, M.A.A. Namboodiri, D. Sugden and J. Barchas, Endocrinol., 113 (1983) 1582.

[40] G. Hernandez, P. Abreu, R. Alonso and C.H. Calzadilla, J. Pineal Res., 8 (1990) 11.

[41] T. Harumi, H. Akutsu and S. Matsushima, J. Chromatogr. B, 675 (1996) 152.

[42] R. Vieira, J. M guez, M. Lema and M. Aldegunde, Anal. Biochem., 205 (1992) 300.

[43] E. Chanut, J. Nguyen-Legros, C. Versaux-Botteri, J.-H. Trouvin and J.-M. Launay, J. Chromatogr. B, 709 (1998) 11.

[44] F. Raynaud and P. P vet, J. Chromatogr., 564 (1991) 103.

[45] E. Tomlinson, T.M. Jefferies and C.M. Riley, J. Chromatogr., 159 (1978) 315.

[46] J. Arendt, J. Neural Transm. Suppl., 21 (1986) 11

[47] R.W. Pelham, C.L. Ralph and I.M. Campbell, Biochem. Biophys. Res. Comm., 46 (1972) 1236.

[48] I. Smith, P.E. Mullen, R.E. Silman, W. Snedden and B.W. Wilson, Nature, 260 (1976) 718.

[49] B.W. Wilson, W. Snedden, R.E. Silman, I. Smith and P. Mullen, Anal. Biochem., 81 (1977) 283.

[50] D.J. Kennaway, R.G. Frith, G. Phillipou, C.D. Matthews and R.F. Seamark, Endocrinol., 101 (1977) 119.

[51] A.J. Lewy and S.P. Markey, Science, 201 (1978) 741.

[52] S.P. Markey, Biomed. Mass Spectrom., 8 (1981) 426.

[53] D.J. Skene, R.M. Leone, I.M. Young and R.E. Silman, Biomed. Mass Spectroscopy, 10 (1983) 655

[54] C.R. Lee and H. Esnaud, Biomed. Environ. Mass Spectrom., 15 (1988) 249.

[55] J.F. Peniston-Bird, W.-L. Di, C.A. Street, A. Kadva, M.A. Stalteri and R.E. Silman, Clin. Chem., 39 (1993) 2242.

[56] E. Bechgaard, K. Lindhardt and L. Martinsen, J. Chromatogr. B, 712 (1998) 177.

[57] E. Kulczykowska and P.M. Iuvone, J. Chromatogr. Sci., 36 (1998) 175.

[58] Y. Sagara, Y. Okatani, S. Yamanaka and T. Kiriyama, J. Chromatogr., 431 (1988) 170.

[59] C.R. Lee and H. Esnaud, Biomed. Environ. Mass Spectrom., 15 (1988) 677.

[60] F. Iinuma, K. Hamase, S. Matsubayashi, M. Takahashi, M. Watanabe and K. Zaitsu, J. Chromatogr. A, 835 (1999) 67.

[61] C. Rocher, L. Bert, F. Robert, J.-H. Trouvin, B. Renaud, C. Jacquot, and A.M. Gardier, Brain Res., 737 (1996) 221.

[62] A.M. Ho and E.S. Yeung, J. Chromatogr. A, 817 (1998) 377.

[63] M.L. Gostkowski, J. Wei and J.B. Shear, Anal. Biochem., 260 (1998) 244.

Figure Legends

Fig. 1 Proposed pathway for tryptophan metabolism in vertebrate pineal gland. Enzymes catalyzing the reactions are noted beside the arrows. Tryptophan hydroxylase (TH); L-aromatic amino acid decarboxylase (AAAD); N-acetyltransferase (NAT), hydroxyindole-O-methyltransferase (HIOMT); monoamine oxidase (MAO); aldehyde reductase (AR) and aldehyde dehydrogenase (AD).

Fig. 2 (A) The triangular area of *Rana pipience* tadpole skin used for melatonin bioassay (upper panel). The five stages of dermal melanophore in the tad pole skin responding to melatonin (lower panel). (B) Response of the dermal melanophores to melatonin concentrations [10].

Fig. 3 Tracing of thin-layer chromatographic separation of indoleamines [12].

5-methoxytryptophol (MTOH); 5-hydroxytryptophol (HTOH); 5-hydroxytryptophan (5HTP) and tryptophan (TP). Other abbreviations are the same as Fig. 1.

Fig. 4 Liquid-phase extraction method of indoleamines from rat pineal gland [14]. Organic phase (OP) and aqueous phase (AP). Other abbreviations are the same as shown in Fig. 1. Recovery rates (%) of each indoleamines are indicated in parentheses.

Fig. 5 Chromatograms of indoleamine standards obtained by gas chromatography (GC) with a flame ionization detector [19]. (a) Isothermal separation of indoleamines at 220 Ž; (b) isothermal separation of silyl derivatives of indoleamines at 220 Ž; (c) isothermal separation of trifluoroacetyl derivatives at 220 Ž; (d) temperature-programmed separation of indoleamines, at 180 Ž for 6 min, to 240 Ž at 15 Ž/min; (e) temperature-programmed separation of silyl derivatives, at 160 Ž for 4 min, to 240 Ž at 10 Ž/min and (f) temperature-programmed separation of trifluoroacetyl derivatives, at 160 Ž for 8 min, to 190 Ž at 10 Ž/min. Tryptamine (I); 5MT (II); N,N-dimethyl tryptamine (III); bufotenin (IV); 5HT (V) and Mel (VI). GC

was performed with a column (glass 120 cm x 0.3 cm I.D.) consisted of 3% SE-30 (methylsilicone), and at a flow rate of 60 ml/min as carrier gas.

Fig. 6 Mass spectra of melatonin (A) and its derivatives, trimethylsilyl melatonin (TMS-Mel) (B), diheptafluorobutyryl melatonin (HFB-Mel) (C), pentafluoropropionyl melatonin (PFP-Mel) (D). For the derivatization of melatonin, bis(trimethylsilyl)trifluoroacetamide (SUPELCO), N-(heptafluoro-n-butyryl)imidazole (Tokyo Kasei) and pentafluoropropionic anhydride (Tokyo Kasei) were used to form TMS-Mel, HFB-Mel, and PFP-Mel, respectively. Analyses were carried out on a Hewlett-Packard 5890J gas-liquid chromatograph connected to JEOL JMS-SX102QQ mass spectrometer. Gas chromatography column was 3 m x 0.32 mm DB-5MS (J & W scientific). The injector was at 280 Ž, and the oven was programmed as follows: at 50 Ž for 1 min; to 80 Ž at 70 Ž / min; at 80 Ž for 2 min; to 280 Ž at 10 Ž / min; to 290 Ž at 70 Ž / min. Under these conditions, the retention time of TMS-Mel, HFB-Mel and PFP-Mel were 20.3, 16.9 and 16.4 min, respectively. Desorption chemical ionization mass spectrometry by direct introduction and positive electron impact mass spectrometry were performed for melatonin and

for melatonin derivatives, respectively. Mass spectra were determined at 70 eV.

Fig. 7 Determination of rat pineal indoleamines by HPLC and fluorometric detection (FD) [37]. (A) Chromatogram of indoleamine standards using mobile phase containing 6.8% acetonitrile; (B) chromatogram of rat pineal gland under same chromatographic conditions with (A); (C) chromatogram of indoleamine standards using mobile phase containing 16% acetonitrile; and (D) chromatogram of rat pineal gland under same chromatographic conditions with (D). 3,4-dihydroxyphenylethylene glycol (DHPG); tyrosine (TYR); 3-methoxy-4-hydroxyphenyl glycol (MHPG); 5-hydroxytryptophan (5HTRP); norepinephrine (NA); 3,4-dihydroxyphenylacetic acid (DOPAC); epinephrine (ADR); 5-hydroxyindole-3-acetic acid (5HIAA); homovanillic acid (HVA); 5-hydroxytryptophol (5HTOL); dopamine (DA); tryptophan (TRP); N-acetyltryptophan (NATRP); 5-methoxytryptophan (5MTRP); 6-hydroxymelatonin (6HMEL); 5-methoxyindole-3-acetic acid (5MIAA); indole-3-acetic acid (IAA); tryptamine (TAM); 5-methoxytryptophol (5MTOL); melatonin (MEL) and tryptophol (TOL). Other abbreviations are the same as Fig. 1. The HPLC condition was described in Table 1. Excitation and emission wavelengths were set at 280 and 340

nm, respectively.

Fig. 8 Determination of rat pineal indoleamines by HPLC and electrochemical detection (ECD) [39]. (A) Chromatograms of pineal glands obtained at 1200h and 2400h using mobile phase containing 10% acetonitrile. 5HT (1); Trp (2); HIAA (3); HTPL (4) and NAS (5). (B) Chromatograms of pineal glands obtained at 1200h and 2400h using mobile phase containing 20% acetonitrile. MIAA (1); indole acetic acid (2); MTPL (3) and Mel (4). The HPLC condition was described in Table 1. The scale changes in detector sensitivity, which started at 100 nA full scale, and was changed at *a* to 20nA full scale, at *b* to 5 nA full scale, and at *c* to 10 nA full scale. The applied potential was 900 mV.

Fig. 9 Effect of sodium 1-octanesulfonate (SOS) concentrations in the HPLC mobile phase on the retention time of 5HT (œ), NAS (£), Mel (¡), Trp (›), HIAA (¢), MTrp ( ), MIAA (¤) and MTPL (ž) [41]. Flow-through time was 1.59 min. The HPLC condition and the composition of the mobile phase (except for SOS) were the described in Table 1. The applied potential was 900 mV.

Fig. 10 Simultaneous determination of 5HT, NAS and Mel on one chromatogram by HPLC-ECD [41]. (A) Chromatogram of indoleamine standards; 1 ng of each standard was injected; (B) chromatogram of a pineal gland extracted from a juvenile golden hamster at night. The HPLC condition was described in Table 1. The applied potential was 900 mV.

Fig. 11 (A) Hydrodynamic voltammogram for 5HT (œ), NAS (£) and Mel (¡). Each point shows the percentage of the current relative to that at 1000 mV of applied potential (asterisk). (B) Background current at each applied potential [41] . The HPLC condition was described in Table 1. The applied potential was 900 mV.

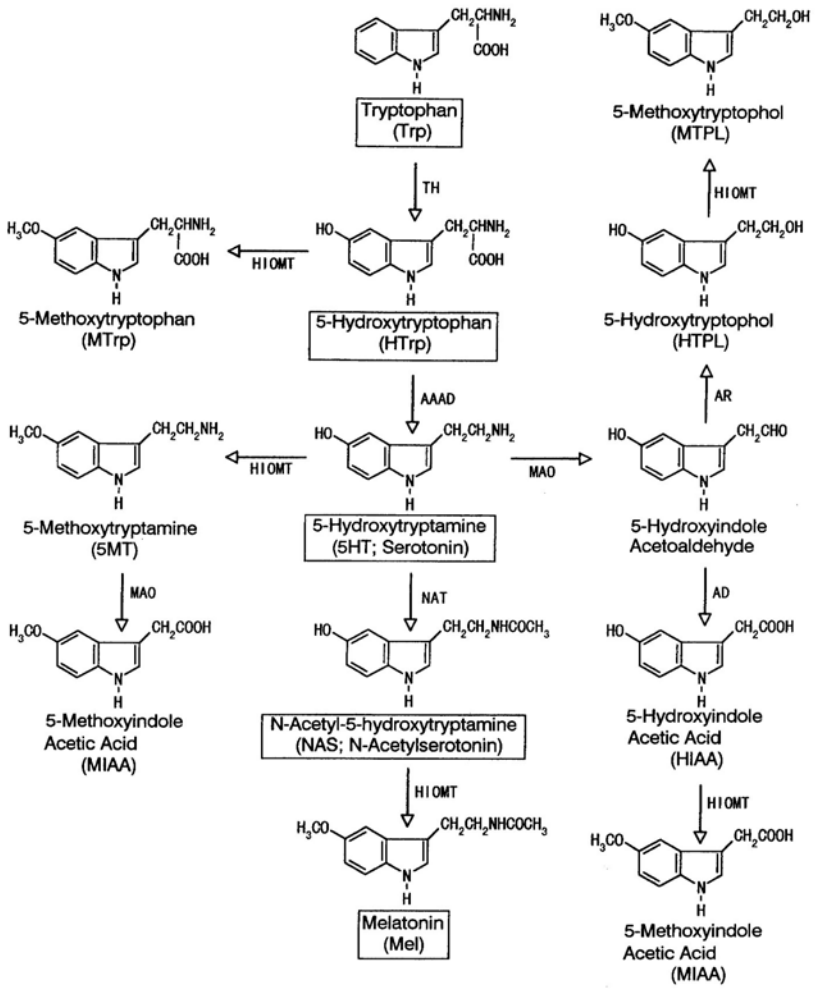
Fig. 12 Determination of plasma Mel by HPLC-ECD [58]. Chromatograms of (A) 500 pg of Mel standard and (B) a sample of blood plasma (1.6 ml equivalent) obtained at midnight. The HPLC condition was described in Table 1. The applied potential was 900 mV.

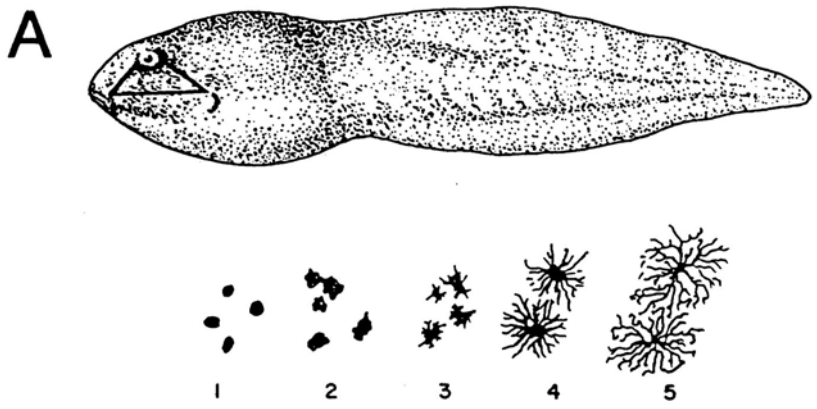
Table 1
Application of reversed-phase HPLC methods for indoleamine determinations

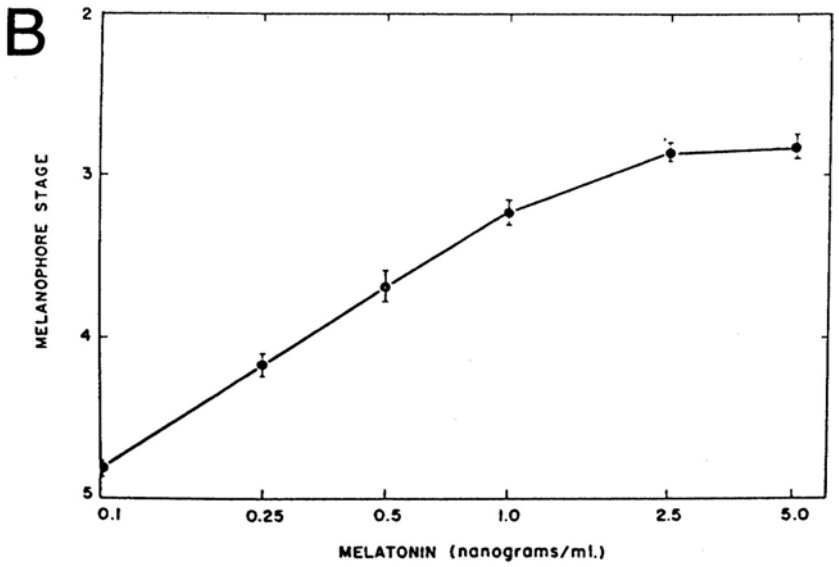
Analyte	Specimen	Analytical column	Mobile phase	Detection method	Flow rate	Reference
Trp, 5HT, HIAA	Rat brain, Rat pineal	µBondapak C18 (300mm x 3.9mm I.D., 10 µm particle)	Methanol-10 mM sodium acetate (12:88), pH 4.25	FD and ECD	?	Anderson, *et. al.* (1981)
HTPL	Rat pineal	µBondapak C18 (300mm x 3.9mm I.D., 10 µm particle)	Methanol-10 mM sodium acetate (15:85), pH 4.25	FD and ECD	?	Anderson, *et. al.* (1982)
Mel	Rat pineal	µBondapak C18 (300mm x 3.9mm I.D., 10 µm particle)	Methanol-10 mM sodium acetate (35:65), pH 4. 25	FD and ECD	?	Anderson, *et. al.* (1982)
Trp, 5HT, HIAA, HTPL	Rat pineal	µBondapak C18 (300mm x 3.9mm I.D., 10 µm particle)	Methanol-10 mM sodium acetate (12:88), pH 4.5	FD	2 ml/min	Anderson, *et. al.* (1982)
Mel	Rat pineal	µBondapak C18 (300mm x 3.9mm I.D., 10 µm particle)	Methanol-10 mM sodium acetate (35:65), pH 4.25	FD	2 ml/min	Anderson, *et. al.* (1982)
5HT, NAS, Mel	Rat Pineal	Zorbax ODS (250 mm x 4.6 mm I.D., 5 µm particle)	Methanol-10 mM sodium acetate (35:65), pH 4.25	FD	1.2 ml/min	Wakabayashi, *et. al.* (1986)
Mel, MIAA, MTPL	Rat pineal	µBondapak C18 (250mm x 4.6mm I.D., 25 µm particle)	Acetonitrile-50 mM phospholic acid, 0.05 M diammonium hydrogen orthophosphate (83:17), pH 5.0	FD	2 ml/min	Chin, *et. al.* (1988)
Trp, HTrp, 5HT, NAS, Mel, HIAA	Rat pineal	Regis ODS-2 (250 mm x 4.6 mm I.D., 5 µm particle)	Acetonitrile-methanol-14.4 mM citric acid, 10 mM sodium acetate, 4 mM sodium octylsulfonate, 1 mM EDTA and 0.25mM dibutylamine phosphate (1:1:8), pH 3.25	FD and ECD	1 ml/min or 1 ml/min to 1.5 ml/min	Chin, *et. al.* (1990)
Trp, HTrp, 5HT, HIAA, HTPL	Rat pineal	Spherisorb ODS II (250 mm x 4.6 mm I.D., 5 µm particle)	Acetonitrile-160 mM ammonium phosphate, 60 mM citric acid, 0.15 MM EDTA, 10 mM dibutylamine, and 6 mM sodium 1-octanesulphonate (6.8:93.2), pH 4.5	FD	1.3 ml/min	Mills, *et. al.* (1991)
Mel, MIAA, 5MT, MTPL	Rat pineal	Spherisorb ODS II (250 mm x 4.6 mm I.D., 5 µm particle)	Acetonitrile-50 mM ammonium phosphate, 50 mM citric acid, 0.15 MM EDTA, 25 mM dibutylamine, and 5 mM sodium 1-octanesulphonate (16:84), pH 5.3	FD	1.3 ml/min	Mills, *et. al.* (1991)

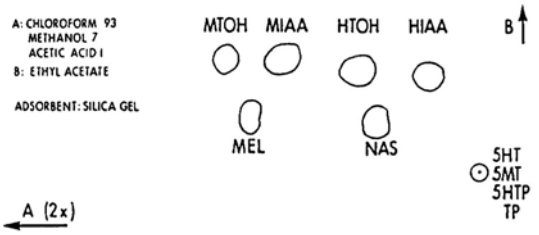
Mel	Rat Pineal	J'sphere ODS-H80 (150 mm x 4.6 mm I.D.,)	Acetonitrile-100 mM sodium phosphate, (12:88), pH 7.0	FD	0.5 ml/min	Iinuma, *et. al.* (1999)
Mel	Rat Pineal Mouse Pineal	TSKgel ODS-80-Ts QA (150 mm x 4.6 mm I.D.)	Acetonitrile-100 mM sodium phosphate, (10:90), pH 7.0	FD	1 ml/min	Hamase, *et. al.* (2000)
			Methanol-10 mM sodium acetate and 2 uM EDTA (30:70), pH 4.0	FD	1 ml/min	Hamase, *et. al.* (2000)
Trp, HTrp, 5HT, HIAA	Rat brain Rat pineal	Vydac 201 TP (250 mm x 3.2 mm I.D., 10 µm particle)	100 mM sodium acetate and 100 mM citric acid, pH 4.1	ECD	0.7 ml/min	Mefford and Barchas (1980)
Mel	Rat pineal	Vydac 201 TP (250 mm x 3.2 mm I.D., 10 µm particle)	Methanol-100 mM sodium acetate and 100 mM citric acid (25:75) , pH 4.1	ECD	0.7 ml/min	Mefford and Barchas (1980)
Trp, 5HT, NAS, HIAA, HTPL	Rat pineal	Ultrasphere (250 mm x 4.6 mm I.D., 5 µm particle)	Acetonitrile- 100 mM acetic acid and 100 mM ammonium acetate (10:90) and 50 mg/l EDTA	ECD	1 ml/min	Mefford, *et. al.* (1983)
Mel, MIAA, MTPL	Rat pineal	Ultrasphere (250 mm x 4.6 mm I.D., 5 µm particle)	Acetonitrile- 100 mM acetic acid and 100 mM ammonium acetate (20:80) and 50 mg/l EDTA	ECD	1 ml/min	Mefford, *et. al.* (1983)
Mel	Rat pineal	uBondapak C18 (10 µm particle)	Acetonitrile-100 mM sodium phosphate and 100 mM EDTA (24:76), pH 5.2	ECD	1.2 ml/min	Hernandez, *et. al.* (1990)
5HT, NAS, Mel	Hamster pineal	Eicompack CA-5ODS (150 mm x 4.6 mm I.D., 5 µm particle)	Methanol-100 mM sodium phosphate, 4 mM sodium 1-octane-sulfonate and 0.1 mM EDTA (25:75), pH 5.0	ECD	1 ml/min	Harumi, *et. al.* (1996)
Mel	Rat pineal, Human plasma	Spherisorb ODS-I (150 mm x 4.6 mm I.D., 5 µm particle)	Acetonitrile-50 mM sodium acetate, 100 mM acetic acid and 0.1 mM EDTA (20:80), pH 4.3	ECD	1 ml/min	Vieira, *et. al.* (1992)
		Spherisorb C_8 (100 mm x 4.6 mm I.D., 3 µm particle)	Acetonitrile-50 mM sodium acetate, 100 mM acetic acid and 0.1 mM EDTA (25:75), pH 4.3	ECD	0.8 ml/min	Vieira, *et. al.* (1992)
Mel	Rat pineal, Rat plasma,	Ultrasphere (150 mm x 4.6 mm I.D., 5 µm particle)	Acetonitrile-100 mM potassium phosphate, 0.5 mM sodium	ECD	1.5 ml/min	Chanut, *et. al.* (1998)

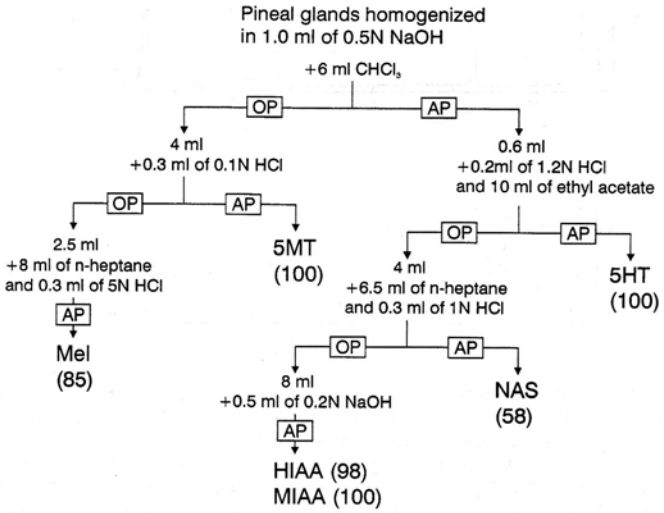
	Rat retina		1-octane sulfonate and 0.01 mM EDTA (20:80), pH 4.7			
Mel, MIAA, 5MT MTPL	Hamster pineal, Hamster brain, Hamster plasma	Beckman C_{18} XL ODS (75 mm x 4.6 mm I.D., 5 µm particle)	Acetonitrile-100 mM sodium phosphate, 100 mM citric acid, 0.15 mM sodium 1-octanesulfonate and 0.1 mM EDTA-triethylamine (14:85.9:0.1), pH4	ECD (dual)	1.3 ml/min	Raynaud and Pevet (1991)
Mel	Human plasma	Hypersil ODS (100 mm x 4.6 mm I.D., 5 µm particle)	Methanol (30% , to 38%, to 80%)	FD	0.5 ml/min to 1 ml/min	Peniston-Bird, *et. al.* (1993)
Mel	Human plasma	Ultrasphere C_{18} (100 mm x 4.6 mm I.D., 5 µm particle)	60% methanol	FD	0.6 ml/min	Kulczykowska and Iuvone (1998)
Mel	Human plasma, cerebrospinal fluid	Supelcosil C_{18} (125 mm x 4.6 mm I.D., 5 µm particle)	Methanol-70 mM potassium dihydrogenphosphate (35:65), pH 3.0	FD	1 ml/min	Toraño, *et al.* (2000)
Trp, HTrp, 5HT, HIAA	Human plasma	IRICA RP-18 (250 mm x 4 mm I.D., 10 µm particle)	Acetonitrile-100 mM sodium acetate, 100 mM citric acid and 0.03 mM EDTA (5:95), pH 4.1	ECD	0.5 ml/min	Sagara, *et. al.* (1988)
Mel	Human plasma	IRICA RP-18 (250 mm x 4 mm I.D., 10 µm particle)	Acetonitrile-100 mM sodium acetate, 100 mM citric acid and 0.03 mM EDTA (20:80), pH 4.1	ECD	0.5 ml/min	Sagara, *et. al.* (1988)
Mel, MIAA, MTPL	Human and mouse bone marrow	C8 (250 mm x 4 mm I.D., 5 µm particle)	Acetonitrile-methanol-100 mM citric acid and 100 mM sodium acetate (10:10:80), pH 4.1	ECD	1 ml/min	Conti, *et. al.* (2000)
Mel	Culture medium of frog retina	Microsorb C18 (150 mm x 4.6 mm I.D., 5 µm particle)	Methanol-50 mM ammonium acetate (33:67), pH 4.25	FD	1 ml/min	Cahill, *et. al.* (1989)
Mel	Silkworm	Superiox ODS-S5 (150 mm x 4.6 mm ID., 5 µm particle)	Methanol-50 mM ammonium acetate (30:70), pH 4.3	FD	1 ml/min	Itoh, *et. al.* (1995)

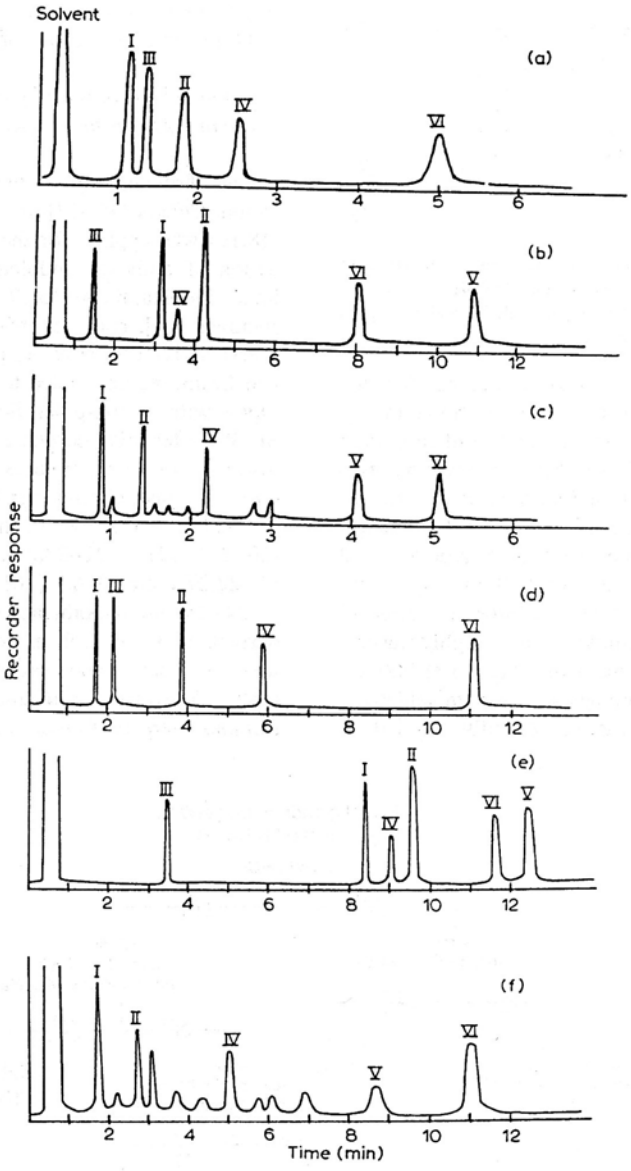

Tryptophan
(Trp)
TH
5-Hydroxytryptophan
(HTrp)
HIOMT
5-Methoxytryptophan
(MTrp)
AAAD
5-Hydroxytryptamine
(5HT; Serotonin)
HIOMT
5-Methoxytryptamine
(5MT)
MAO
5-Methoxyindole
Acetic Acid
(MIAA)
NAT
N-Acetyl-5-hydroxytryptamine
(NAS; N-Acetylserotonin)
HIOMT
Melatonin
(Mel)
MAO
5-Hydroxyindole
Acetoaldehyde
AR
5-Hydroxytryptophol
(HTPL)
HIOMT
5-Methoxytryptophol
(MTPL)
AD
5-Hydroxyindole
Acetic Acid
(HIAA)
HIOMT
5-Methoxyindole
Acetic Acid
(MIAA)

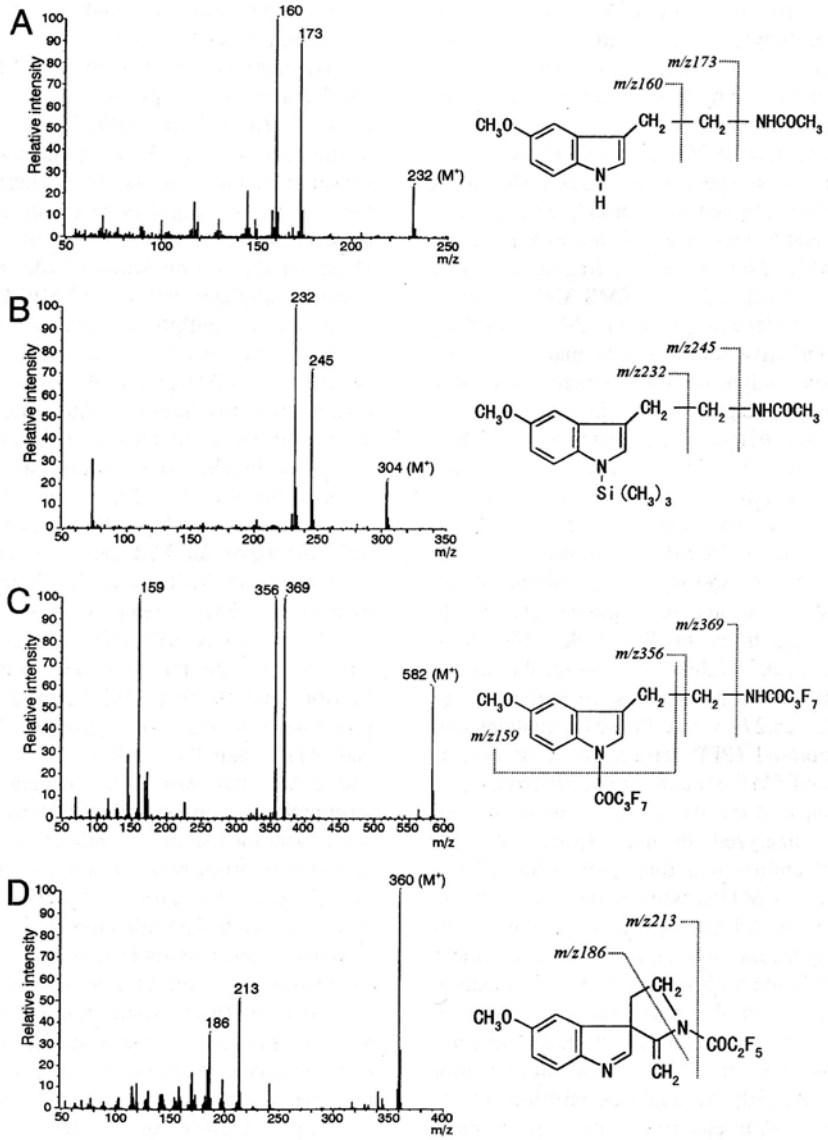

A
1
2
3
4
5

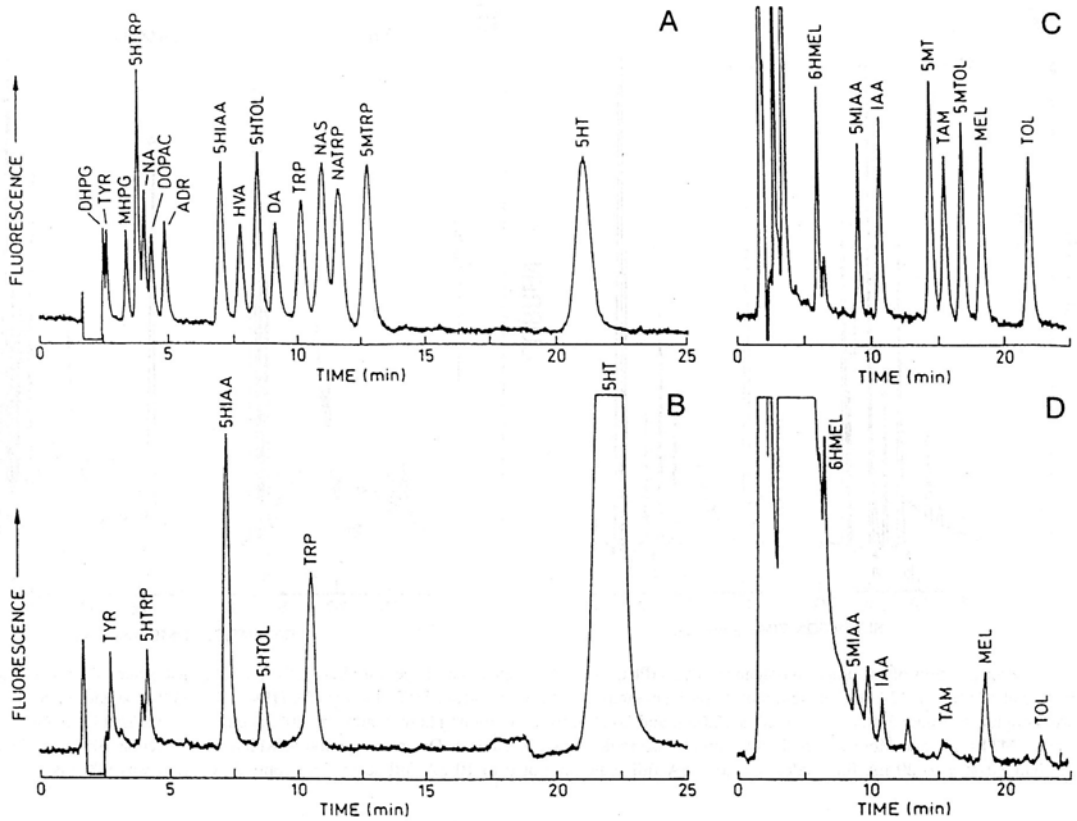

B
MELANOPHORE STAGE
2
3
4
5
0.1
0.25
0.5
1.0
2.5
5.0
MELATONIN (nanograms/ml.)

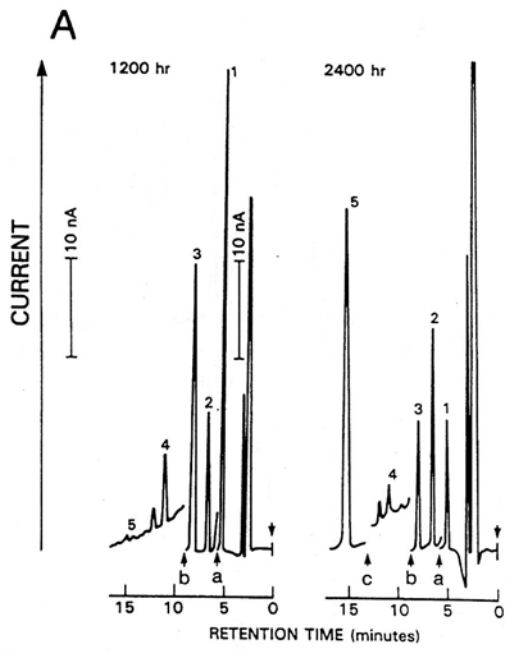

A: CHLOROFORM 93
METHANOL 7
ACETIC ACID 1
B: ETHYL ACETATE
ADSORBENT: SILICA GEL
MTOH
MIAA
HTOH
HIAA
B
MEL
NAS
5HT
5MT
5HTP
TP
A (2x)

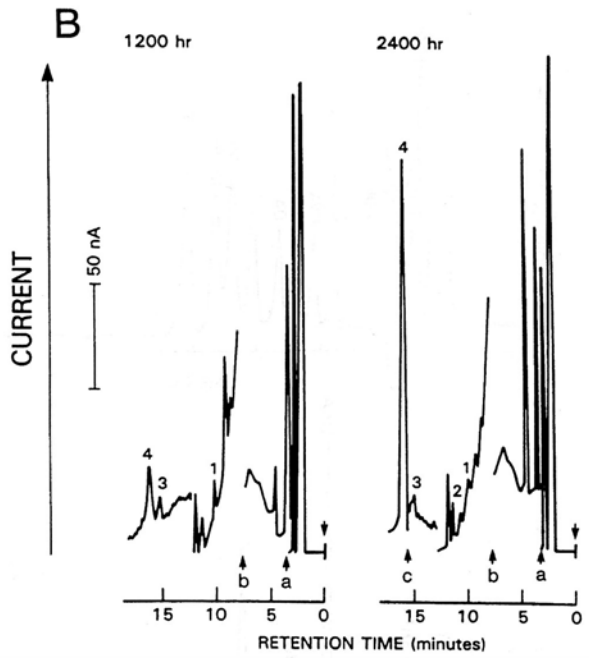

Pineal glands homogenized
in 1.0 ml of 0.5N NaOH
+6 ml $CHCl_3$
OP
AP
4 ml
+0.3 ml of 0.1N HCl
0.6 ml
+0.2ml of 1.2N HCl
and 10 ml of ethyl acetate
OP
AP
2.5 ml
+8 ml of n-heptane
and 0.3 ml of 5N HCl
AP
Mel
(85)
5MT
(100)
OP
AP
4 ml
+6.5 ml of n-heptane
and 0.3 ml of 1N HCl
5HT
(100)
OP
AP
8 ml
+0.5 ml of 0.2N NaOH
AP
HIAA (98)
MIAA (100)
NAS
(58)

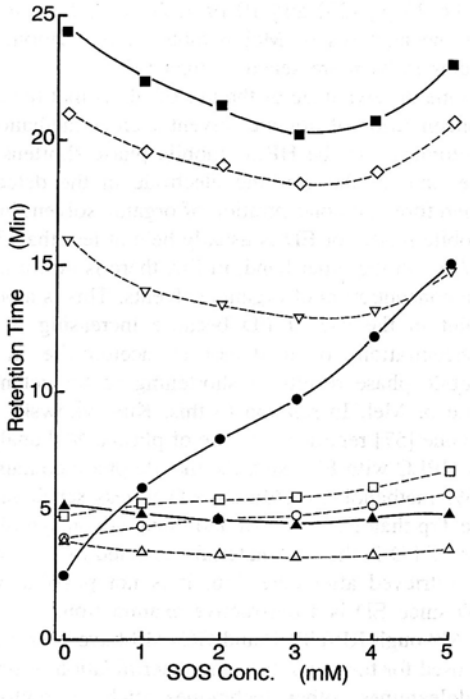

Solvent
(a)
(b)
(c)
(d)
(e)
(f)
Recorder response
Time (min)

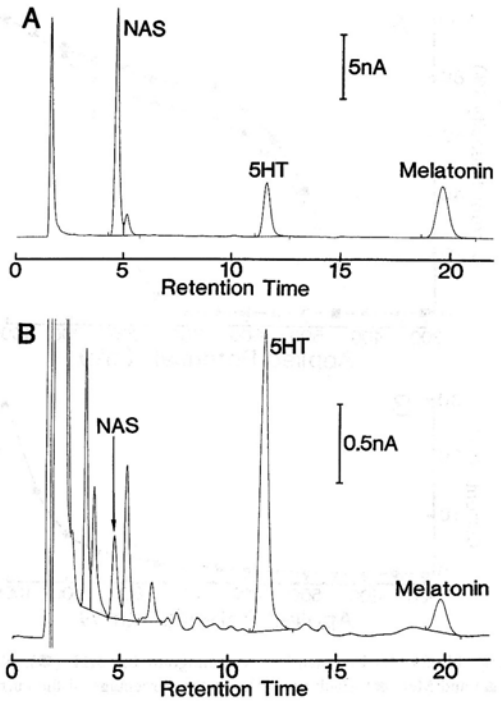

A
Relative intensity
160
173
232 (M+)
m/z
m/z173
m/z160
CH3O
CH2
CH2
NHCOCH3
N
H
B
Relative intensity
232
245
304 (M+)
m/z
m/z245
m/z232
CH3O
CH2
CH2
NHCOCH3
N
Si (CH3)3
C
Relative intensity
159
356
369
582 (M+)
m/z
m/z369
m/z356
m/z159
CH3O
CH2
CH2
NHCOC3F7
N
COC3F7
D
Relative intensity
360 (M+)
213
186
m/z
m/z213
m/z186
CH3O
CH2
N
COC2F5
CH2
N

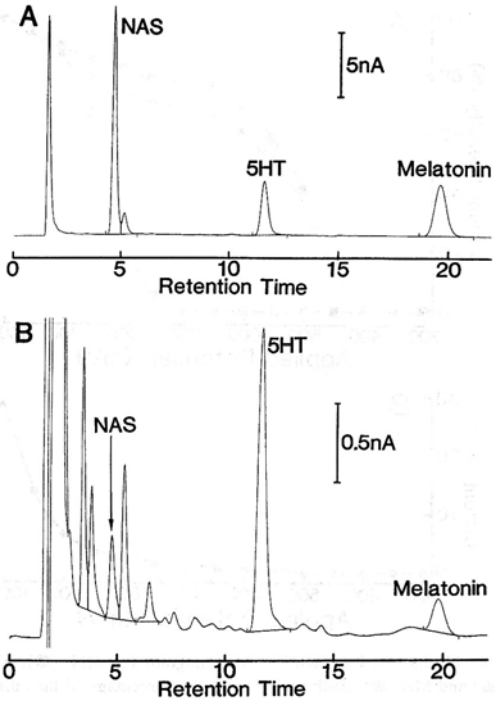

A
FLUORESCENCE
DHPG
TYR
MHPG
5HTRP
NA
DOPAC
ADR
5HIAA
HVA
5HTOL
DA
TRP
NAS
NATRP
5MTRP
5HT
TIME (min)
C
6HMEL
5MIAA
IAA
5MT
TAM
5MTOL
MEL
TOL
TIME (min)
B
FLUORESCENCE
TYR
5HTRP
5HIAA
5HTOL
TRP
5HT
TIME (min)
D
6HMEL
5MIAA
IAA
TAM
MEL
TOL
TIME (min)

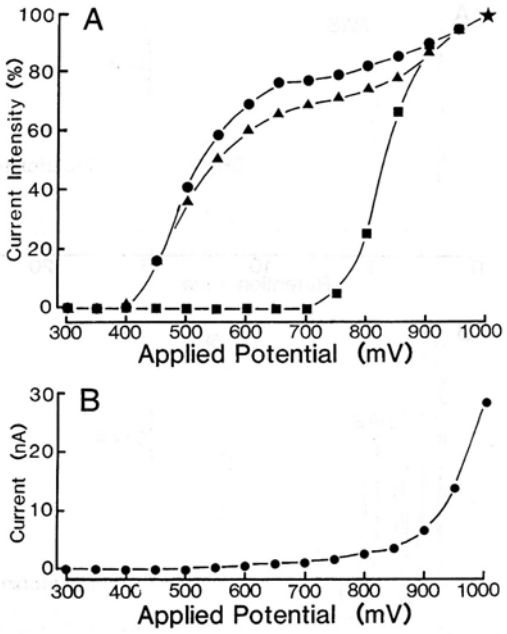

A
1200 hr
2400 hr
CURRENT
10 nA
10 nA
RETENTION TIME (minutes)

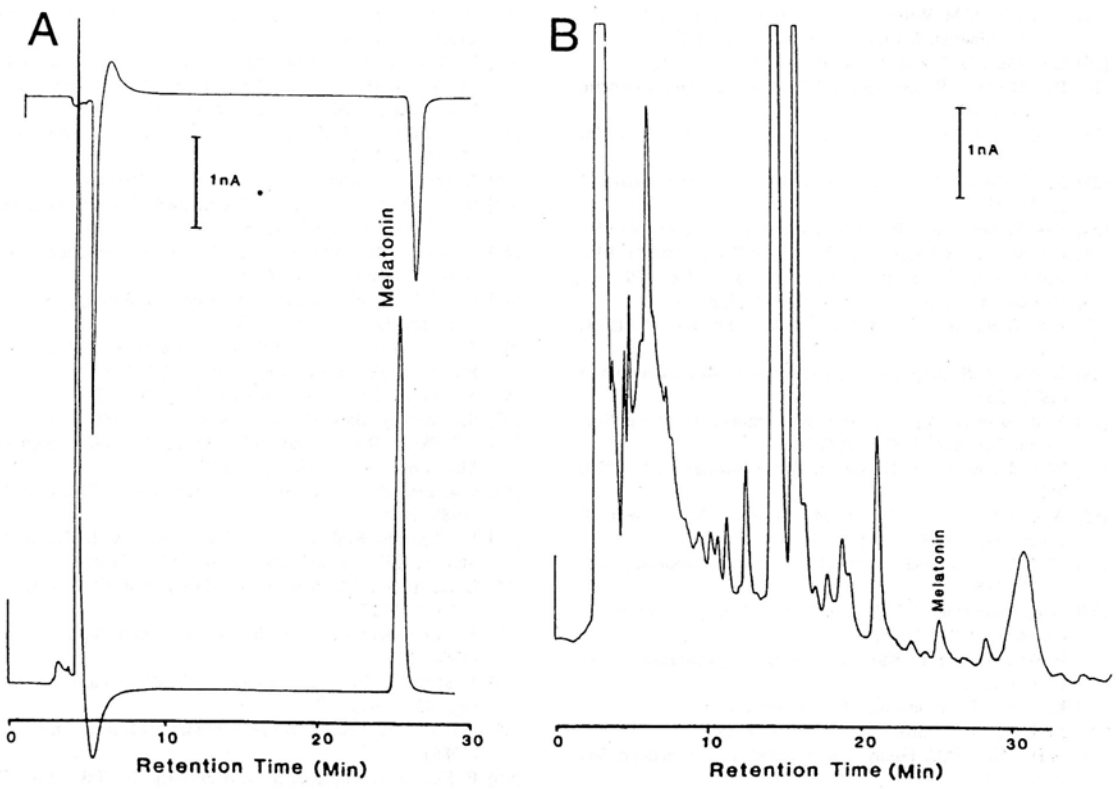

B
1200 hr
2400 hr
CURRENT
50 nA
RETENTION TIME (minutes)


Retention Time (Min)
SOS Conc. (mM)


A
NAS
5nA
5HT
Melatonin
Retention Time
B
5HT
NAS
0.5nA
Melatonin
Retention Time


A
NAS
5nA
5HT
Melatonin
0
5
10
15
20
Retention Time
B
5HT
NAS
0.5nA
Melatonin
0
5
10
15
20
Retention Time


A
100
80
60
40
20
0
Current Intensity (%)
300
400
500
600
700
800
900
1000
Applied Potential (mV)
B
30
20
10
0
Current (nA)
300
400
500
600
700
800
900
1000
Applied Potential (mV)


A
1nA
Melatonin
0
10
20
30
Retention Time (Min)
B
1nA
Melatonin
0
10
20
30
Retention Time (Min)