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Review

Separation and assay methods for melatonin and its precursors

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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: Reviews; Melatonin; Indoleamines

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1. Introduction

Melatonin is an indoleamine hormones 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 [1–4]. Melatonin was first isolated from the pineal gland as a potent frog skin-lightening agent, and was characterized as *N*-acetyl-5-methoxy-

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tryptamine in 1959 by Lerner and co-workers [5,6]. Thereafter, many methods were developed and improved for determination of melatonin in the pineal gland, blood plasma and other organs. At the same time, quantitative analyses of the pineal indoleamines were started because the researchers noticed that the contents of melatonin, its precursors, and its related indoleamines in the most mammalian pineal gland show clear circadian changes. To determine the content of each indoleamine, methods for separation using chromatographic techniques were also developed and improved. At present, it is well-known that melatonin is present even in invertebrates [7] and plants [8]. This review will focus on the methods of qualitative and quantitative analyses of melatonin and its related indoleamines in biological materials.

2. Melatonin biosynthesis and indoleamine metabolism in the pineal gland

The metabolic pathway of melatonin and indoleamine 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). A methyl group from *S*-adenosylmethionine is transferred to NAS by hydroxyindole-*O*-methyltransferase (HIOMT), and finally NAS is converted to 5-methoxy-*N*-acetyltryptamine, or melatonin (Mel). Mel is highly hydrophobic because the 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 oxidase (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 the contents of Mel and NAS in the dark period is due to the increase of SNAT activity and the elevation of SNAT gene expression [9]. On the other hand, the content of 5HT in the pineal gland shows circadian changes opposite to the Mel and NAS rhythm. These circadian changes of 5HT content in the pineal gland may relate to its cyclic production from Trp, metabolism to NAS, and release from the pinealocytes. The contents of HIAA, MIAA, HTPL and MTPL also 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 method was developed based on the experimental fact that Mel induces the aggregation of the melanin granules within the living dermal melanophore (Fig. 2A). By assessing the dermal melanophore response 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. Planar techniques

Lerner and co-workers first demonstrated that Mel is *N*-acetyl-5-methoxytryptamine [5,6]. Mel obtained from bovine pineal gland and synthetic *N*-acetyl-5-

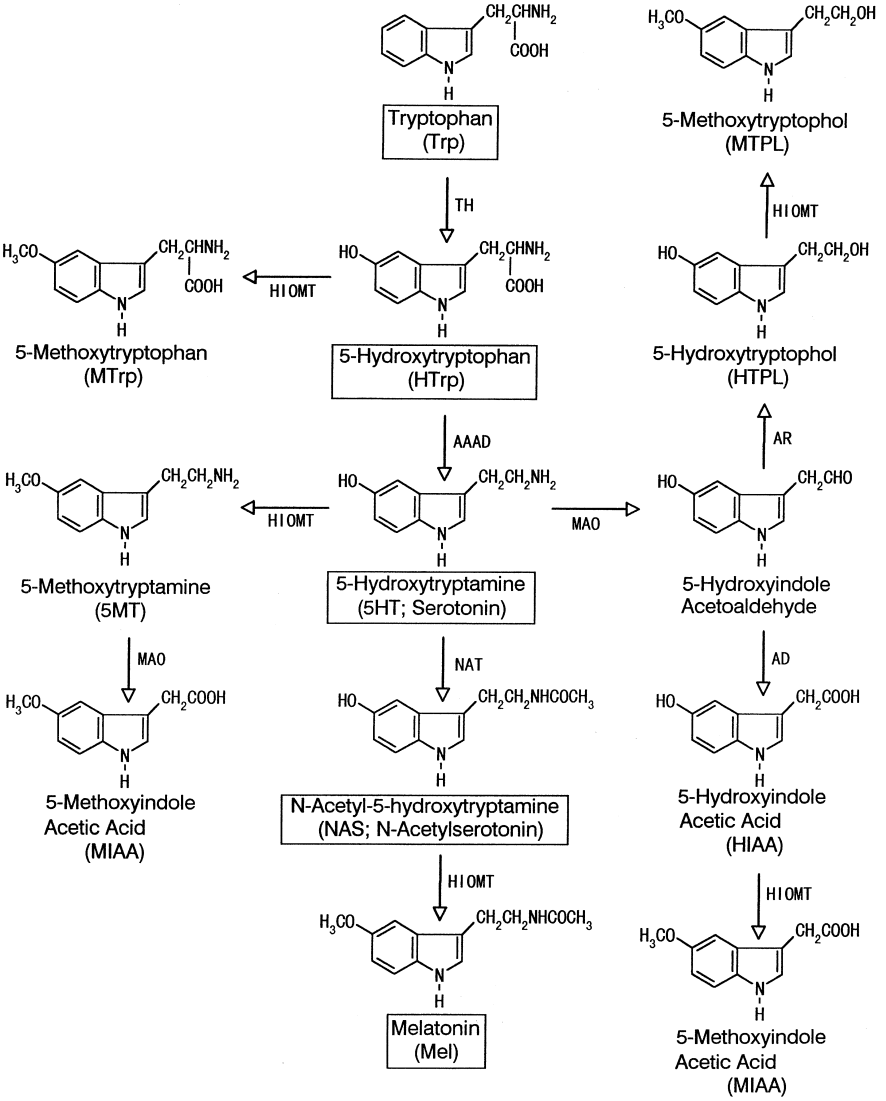

Fig. 1. Proposed pathway for tryptophan metabolism in vertebrate pineal gland. Enzymes catalyzing the reactions are noted beside the arrows. Tryptophan hydroxylase (TH); L-aromatic amino acid decarboxylase (AAAD); *N*-acetyltransferase (NAT); hydroxyindole-*O*-methyltransferase (HIOMT); monoamine oxidase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

methoxytryptamine were proven to be 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 R_F 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 an ultraviolet lamp or by colored spot with six different dyes. Klein and Notides [12] employed a two-dimensional thin-layer chromatography for the

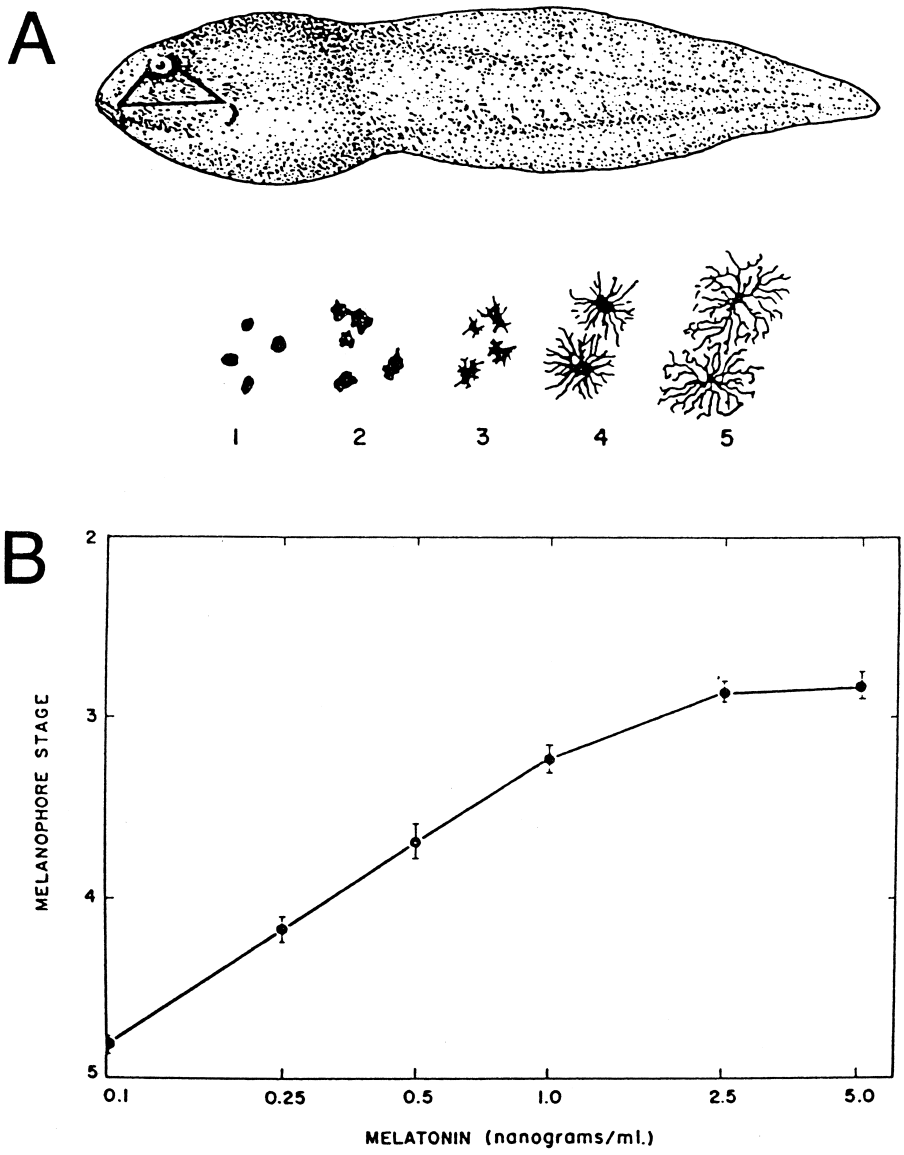

Fig. 2. (A) The triangular area of *Rana pipience* tadpole skin used for melatonin bioassay (upper panel). The five stages of dermal melanophore in the tadpole skin responding to melatonin (lower panel). (B) Response of the dermal melanophores to melatonin concentrations [10].

separation of Mel, NAS, HTPL, HIAA, MIAA and MTPL. In their case, 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 the application of column chromatographic techniques for separations of indoleamine, liquid-

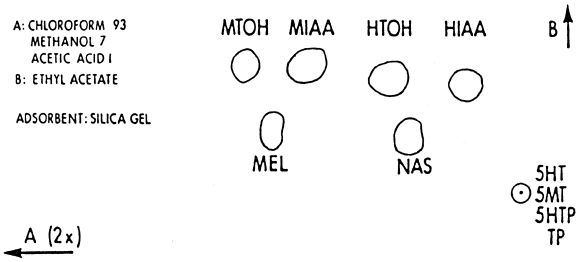

Fig. 3. Tracing of thin-layer chromatographic separation of indoleamines [12]. 5-Methoxytryptophol (MTOH); 5-hydroxytryptophol (HTOH); 5-hydroxytryptophan (5HTP) and tryptophan (TP). Other abbreviations as in Fig. 1.

phase extraction method 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, 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 3 *M* HCl. Miller and Maickel reported that the indoleamines yielded highly fluorescent compounds at 470 nm when excited at 360 nm after reaction of indoleamines with *o*-phthalaldehyde [14]. This technique was successfully applied to measurement of the contents of 5HT, NAS, Mel and 5MT in the pineal gland of dog and rat [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], their acetylated derivatives [16], and their trimethylsilyl and trifluoroacetyl derivatives (Fig. 5) [19] were analyzed. Cole and Crank recommended to perform gas chromatography with a non-polar liquid phase (for example SE-30) after silyl derivatization of indoleamines to lower 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

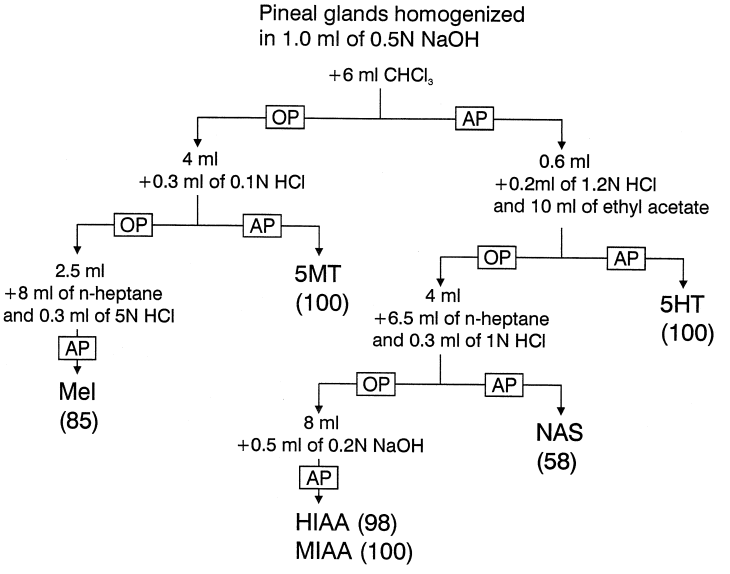

Fig. 4. Liquid-phase extraction method of indoleamines from rat pineal gland [14]. Organic phase (OP) and aqueous phase (AP). Other abbreviations as in Fig. 1. Recovery rates (%) of each indoleamine are indicated in parentheses.

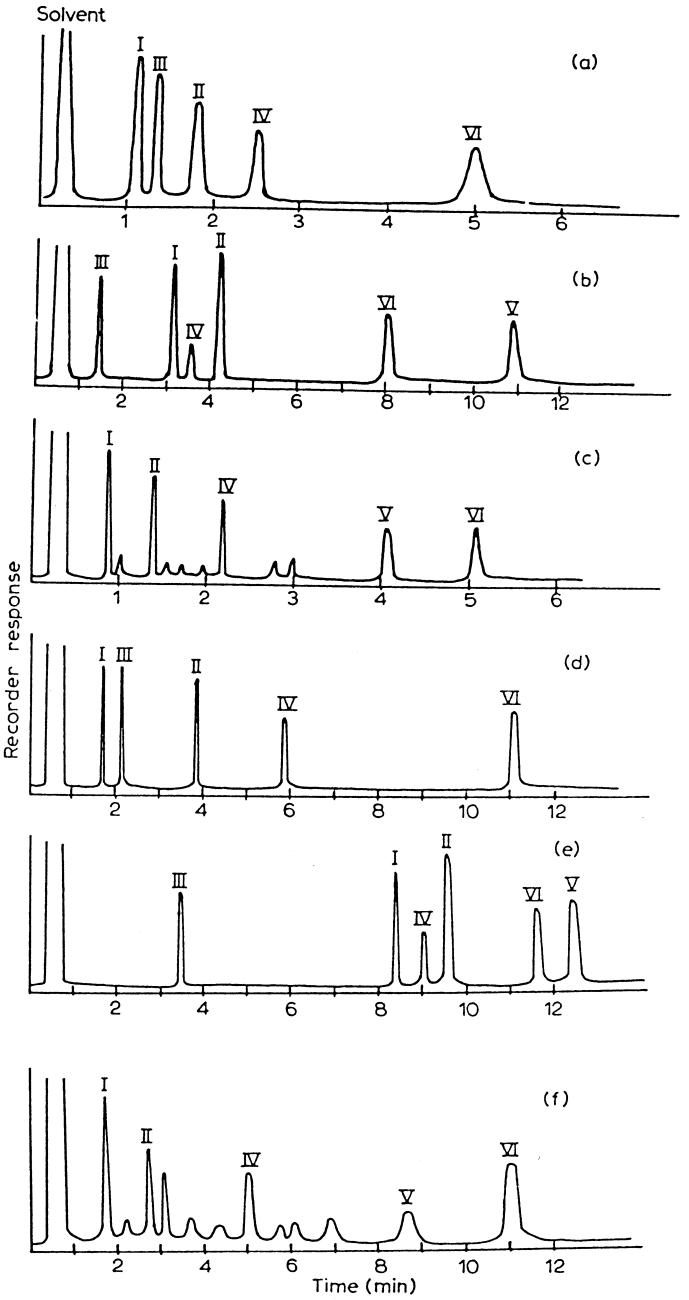

Fig. 5. Gas chromatograms of authentic indoleamine obtained using a flame ionization detector [19]. (a) Isothermal separation of indoleamines at 220°C; (b) isothermal separation of silyl derivatives of indoleamines at 220°C; (c) isothermal separation of trifluoroacetyl derivatives at 220°C; (d) temperature-programmed separation of indoleamines, at 180°C for 6 min, to 240°C at 15°C/min; (e) temperature-programmed separation of silyl derivatives, at 160°C for 4 min, to 240°C at 10°C/min and (f) temperature-programmed separation of trifluoroacetyl derivatives, at 160°C for 8 min, to 190°C at 10°C/min. Tryptamine (I); 5MT (II); *N,N*-dimethyl tryptamine (III); bufotenin (IV); 5HT (V) and Mel (VI). Gas chromatography was performed with a column (glass 120 cm×0.3 cm I.D.) consisting of 3% SE-30 (methylsilicone), and at carrier gas flow-rate of 60 ml/min.

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 β and γ 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 [30] analyzed the mass spectra of eight synthetic indoleamines including Mel, NAS, HIAA, MIAA, HTPL and MTPL without derivatization by direct introduction. All the eight indoleamines yielded a single molecular ion and abundant fragment ions due to the β and γ 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 isocratic 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 simple sample preparation, many investigators used the system for analysis of Mel and indoleamines in the pineal gland [31–44]. Vitale et al. [31] reported that HPLC with a normal-phase column and an organic non-polar mobile phase is a sensitive method for the Mel determination. However, many investigators recommended HPLC with reversed-phase columns using 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 chromatography. However, in the most cases, repeated HPLC with different mobile phases is required to obtain better resolution of pineal indoleamines since the pineal gland contains a wide range of polar and non-polar indoleamines. Anderson and co-workers reported the use of HPLC mobile phases containing 12% methanol for Trp, 5HT, HIAA and NAS, and 35% methanol for Mel determinations [32,33]. On the other hand, Mills et al. [37] recommend 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). Mefford and Barchas [38] reported that the mobile phase without methanol is good for Trp, 5HT, HIAA and HTPL, and that with 10% methanol for MTrp and NAS, and with 25% methanol for Mel determinations. Furthermore, Mefford et al. [39] reported that the use of a mobile phase containing 10% acetonitrile gives better resolution for Trp and hydroxyindoles, and 20% acetonitrile for methoxyindoles and NAS determinations (Fig. 8).

When a polar or ionized solute is mixed with an ion charged opposite or a counter ion, an ion-pair is formed between the solute ion and the counter ion, and the polarity of the resulting complex is decreased. 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, alkylsulfonates, inorganic ions and surface-active ions were often utilized for the separation of polar samples by RP-HPLC [45]. Raynaud and Pévet [44] used triethylamine (TEA) and sodium 1-octanesulfonate (SOS) as ion pairing agents for the

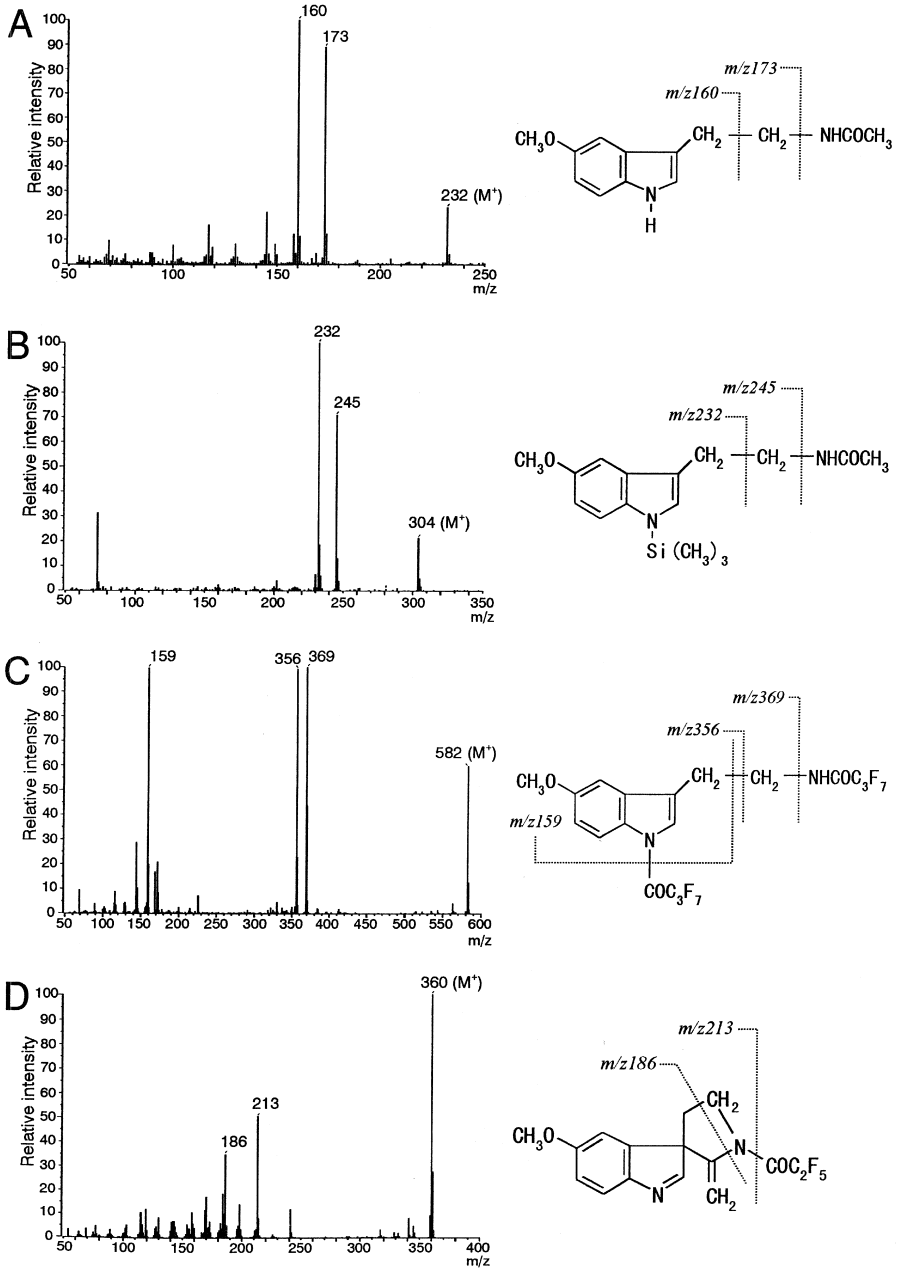

Fig. 6. Mass spectra of (A) melatonin, (B) trimethylsilyl melatonin (TMS-Mel), (C) diheptafluorobutyryl melatonin (HFB-Mel) and (D) pentafluoropropionyl melatonin (PFP-Mel). For the derivatization of melatonin, bis(trimethylsilyl)trifluoroacetamide (Supelco), *N*-(heptafluoro-*n*-butyryl)imidazole (Tokyo 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×0.32 mm DB-5MS (J&W Scientific). The injector temperature was set at 280°C, and the oven was programmed as follows: at 50°C for 1 min; to 80°C at 70°C/min; at 80°C for 2 min; to 280°C at 10°C/min; to 290°C at 70°C/min. Under these conditions, the retention times 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.

Table 1
Reversed-phase HPLC methods for indoleamine determinations

Analyte	Specimen	Analytical column	Mobile phase	Detection method	Flow-rate	Ref.
Trp, 5HT, HIAA	Rat brain, rat pineal	μBondapak C_{18} (300 mm× 3.9 mm I.D., 10 μm particle)	Methanol–10 m*M* sodium acetate (12:88), pH 4.25	FD and ED	Not described	[32]
HTPL	Rat pineal	μBondapak C_{18} (300 mm× 3.9 mm I.D., 10 μm particle)	Methanol–10 m*M* sodium acetate (15:85), pH 4.25	FD and ED	Not described	[32]
Mel	Rat pineal	μBondapak C_{18} (300 mm× 3.9 mm I.D., 10 μm particle)	Methanol–10 m*M* sodium acetate (35:65), pH 4.25	FD and ED	Not described	[32]
Trp, 5HT, HIAA, HTPL	Rat pineal	μBondapak C_{18} (300 mm× 3.9 mm I.D., 10 μm particle)	Methanol–10 m*M* sodium acetate (12:88), pH 4.5	FD	2 ml/min	[33]
Mel	Rat pineal	μBondapak C_{18} (300 mm× 3.9 mm I.D., 10 μm particle)	Methanol–10 m*M* sodium acetate (35:65), pH 4.25	FD	2 ml/min	[33]
5HT, NAS, Mel	Rat pineal	Zorbax ODS (250 mm× 4.6 mm I.D., 5 μm particle)	Methanol–10 m*M* sodium acetate (35:65), pH 4.25	FD	1.2 ml/min	[34]
Mel, MIAA, MTPL	Rat pineal	μBondapak C_{18} (250 mm× 4.6 mm I.D., 25 μm particle)	Acetonitrile–50 m*M* phospholic acid and 50 m*M* diammonium hydrogen orthophosphate (83:17), pH 5	FD	2 ml/min	[35]
Trp, HTrp, 5HT, NAS, Mel, HIAA	Rat pineal	Regis ODS-2 (250 mm× 4.6 mm I.D., 5 μm particle)	Acetonitrile–methanol–14.4 m*M* citric acid, 10 m*M* sodium acetate, 0.25 m*M* dibutylamine phosphate, 4 m*M* sodium octylsulfonate and 1 m*M* EDTA (1:1:8), pH 3.25	FD and ED	1 ml/min or 1–1.5 ml/min	[36]
Trp, HTrp, 5HT, HIAA, HTPL	Rat pineal	Spherisorb ODS II (250 mm× 4.6 mm I.D., 5 μm particle)	Acetonitrile–160 m*M* ammonium phosphate, 60 m*M* citric acid, 10 m*M* dibutylamine, 6 m*M* sodium 1-octanesulfonate and 0.15 m*M* EDTA (6.8:93.2), pH 4.5	FD	1.3 ml/min	[37]
Mel, MIAA, 5MT, MTPL	Rat pineal	Spherisorb ODS II (250 mm× 4.6 mm I.D., 5 μm particle)	Acetonitrile–50 m*M* ammonium phosphate, 50 m*M* citric acid, 10 m*M* dibutylamine, 6 m*M* sodium 1-octanesulfonate and 0.15 m*M* EDTA (16:84), pH 5.3	FD	1.3 ml/min	[37]
Trp, HTrp, 5HT, HIAA	Rat brain, rat pineal	Vydac 201 TP (250 mm× 3.2 mm I.D., 10 μm particle)	100 m*M* Sodium acetate and 100 m*M* citric acid, pH 4.1	ED	0.7 ml/min	[38]
Mel	Rat pineal	Vydac 201 TP (250 mm× 3.2 mm I.D., 10 μm particle)	Methanol–100 m*M* sodium acetate and 100 m*M* citric acid (25:75), pH 4.1	ED	0.7 ml/min	[38]
Trp, 5HT, NAS, HIAA, HTPL	Rat pineal	Ultrasphere (250 mm× 4.6 mm I.D., 5 μm particle)	Acetonitrile–100 m*M* acetic acid and 100 m*M* ammonium acetate (10:90) and 50 mg/l EDTA, pH 5.1	ED	1 ml/min	[39]
Mel, MIAA, MTPL	Rat pineal	Ultrasphere (250 mm× 4.6 mm I.D., 5 μm particle)	Acetonitrile–100 m*M* acetic acid and 100 m*M* ammonium acetate (20:80) and 50 mg/l EDTA, pH 5.1	ED	1 ml/min	[39]
Mel	Rat pineal	Bondapak C_{18} (10 μm particle)	Acetonitrile–100 m*M* sodium phosphate and 100 m*M* EDTA (24:76), pH 5.2	ED	1.2 ml/min	[40]
5HT, NAS, Mel	Hamster pineal	Eicompack CA-5ODS (150 mm× 4.6 mm I.D., 5 μm particle)	Methanol–100 m*M* sodium phosphate, 4 m*M* sodium 1-octanesulfonate and 0.1 m*M* EDTA (25:75), pH 5.0	ED	1 ml/min	[41]

Table 1. Continued

Analyte	Specimen	Analytical column	Mobile phase	Detection method	Flow-rate	Ref.
Mel	Rat pineal, human plasma	Spherisorb ODS-I (150 mm× 4.6 mm I.D., 5 μm particle)	Acetonitrile–50 m*M* sodium acetate, 100 m*M* acetic acid and 0.1 m*M* EDTA (20:80), pH 4.3	ED	1 ml/min	[42]
		Spherisorb C_8 (100 mm× 4.6 mm I.D., 3 μm particle)	Acetonitrile–50 m*M* sodium acetate, 100 m*M* acetic acid and 0.1 m*M* EDTA (25:75), pH 4.3	ED	0.8 ml/min	[42]
Mel	Rat pineal, rat plasma, rat retina	Ultrasphere (150 mm× 4.6 mm I.D., 5 μm particle)	Acetonitrile–100 m*M* potassium phosphate, 0.5 m*M* sodium 1-octanesulfonate and 0.01 m*M* EDTA (20:80), pH 4.7	ED	1.5 ml/min	[43]
Mel, MIAA, 5MT MTP	Hamster pineal, hamster brain, hamster plasma	Beckman C_{18} XL ODS (75 mm× 4.6 mm I.D., 5 μm particle)	Acetonitrile–100 m*M* sodium phosphate, 100 m*M* citric acid, 0.15 m*M* sodium 1-octanesulfonate and 0.1 m*M* EDTA–triethylamine (14:85.9:0.1), pH 4.3	ED (dual)	1.3 ml/min	[44]
Mel	Human plasma	Hypersil ODS (100 mm× 4.6 mm I.D., 5 μm particle)	Methanol (30% to 38% to 80%)	FD	0.5–1 ml/min	[55]
Mel	Human plasma	Ultrasphere C_{18} (100 mm× 4.6 mm I.D., 5 μm particle)	60% Methanol	FD	0.6 ml/min	[57]
Trp, HTrp, 5HT, HIAA	Human plasma	IRICA RP-18 (250 mm× 4 mm I.D., 10 μm particle)	Acetonitrile–100 m*M* sodium acetate, 100 m*M* citric acid and 0.03 m*M* EDTA (5:95), pH 4.1	ED	0.5 ml/min	[58]
Mel	Human plasma	IRICA RP-18 (250 mm× 4 mm I.D., 10 μm particle)	Acetonitrile–100 m*M* sodium acetate, 100 m*M* citric acid and 0.03 m*M* EDTA (20:80), pH 4.1	ED	0.5 ml/min	[58]

determination of 5MT, MIAA, MTPL and Mel. Addition of TEA (0.1%) to the RP-HPLC mobile phase results in an increase in the retention time of MIAA, but a slight decrease in the retention time of 5MT. Contrastingly, addition of SOS (0.2 m*M*) increases the retention time of 5MT, but slightly decreases the retention time of MIAA. However, the retention times of Mel and MTPL are not affected by 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 m*M* 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 increases with the SOS concentrations. On the other hand, increasing the SOS concentration from 0 to 3 m*M* 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 m*M*). Fig. 10 shows typical HPLC chromatograms of authentic 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 including 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: fluorometric detection (FD) (Fig. 7) [31–37] and electrochemical detection (ED) (Fig. 8) [32,33,36,38–44]. In FD of the pineal indoleamines, the following set of excitation and emission wavelengths is usually used 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 ED of indoleamines, the setting of the applied potential is important. Usually,

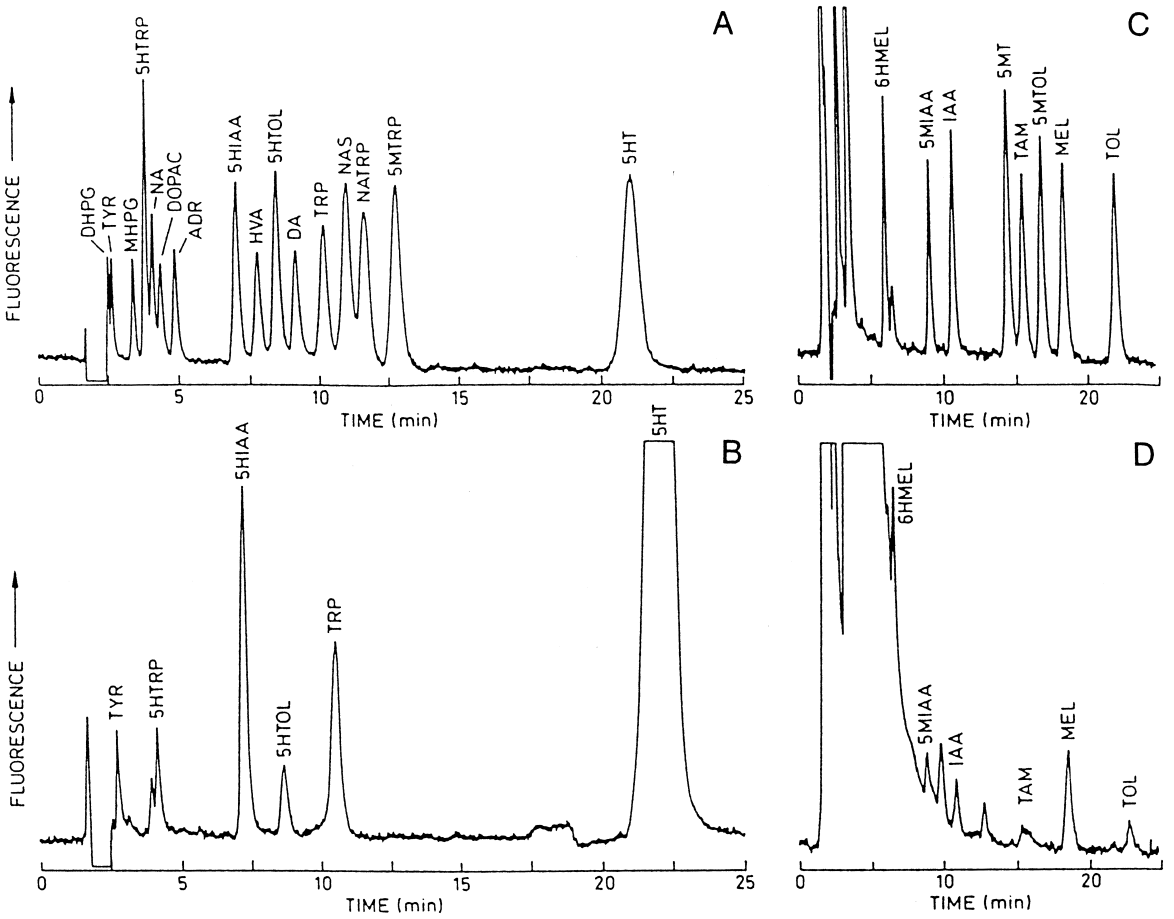

Fig. 7. Determination of rat pineal indoleamines by HPLC with fluorometric detection (FD) [37]. (A) Chromatogram of authentic indoleamine using mobile phase containing 6.8% acetonitrile; (B) chromatogram of rat pineal gland under same chromatographic conditions as (A); (C) chromatogram of authentic indoleamine using mobile phase containing 16% acetonitrile and (D) chromatogram of rat pineal gland under same chromatographic conditions as (C). 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 as in Fig. 1. The HPLC conditions are described in Table 1. Excitation and emission wavelengths used were set at 280 and 340 nm, respectively.

an applied potential greater than 800 mV gives the higher sensitivity for Mel detection (Fig. 11A). However, a higher potential causes an elevation of the baseline noise (Fig. 11B). Therefore, the applied potential for Mel determination is usually set at 700 mV [32,33], 850 mV [36], 900 mV (Fig. 8, 10) [38–43], and 1.25 V [44]. A dual ED 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].

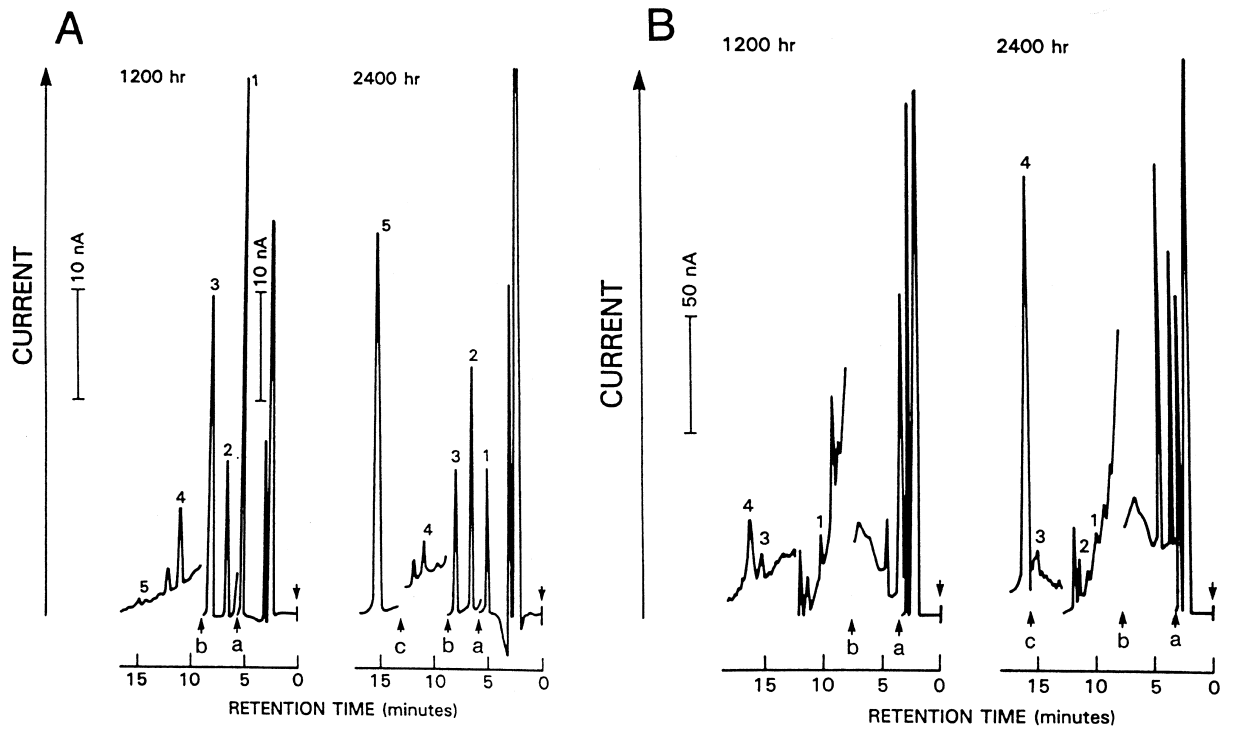

Fig. 8. Determination of rat pineal indoleamines by HPLC with electrochemical detection (ED) [39]. (A) Chromatograms of pineal glands obtained at 12.00 h and 24.00 h using a mobile phase containing 10% acetonitrile. 5HT (1); Trp (2); HIAA (3); HTPL (4) and NAS (5). (B) Chromatograms of pineal glands obtained at 12.00 h and 24.00 h using a mobile phase containing 20% acetonitrile. MIAA (1); indole acetic acid (2); MTPL (3) and Mel (4). The HPLC conditions are described in Table 1. Detector sensitivity, which was started at 100 nA full scale, was changed at *a* to 20 nA full scale, at *b* to 5 nA full scale, and at *c* to 10 nA full scale. The applied potential was 900 mV.

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. However, normal daytime plasma Mel levels are extremely low (<43 pmol/l; 10 pg/ml). Therefore, a specific and sensitive assay method such as radioimmunoassay (RIA) is necessary. RIA has been widely used for the measurement of plasma Mel levels because of its high sensitivity and easiness of the measurement of large numbers of samples at a time. Several commercial kits are available for measurement of the plasma Mel levels [46]. On the other hand, GC–MS [47–54] and RP-HPLC with either FD [55–57] or ED (Fig. 12) [42–44,58] are also useful for the Mel analysis in the blood plasma.

In the early work on 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 of Mel in the pineal gland, Mel in the gland is extracted with an acid such as 10% perchloric acid. However, for the determination of Mel in blood plasma, several prior

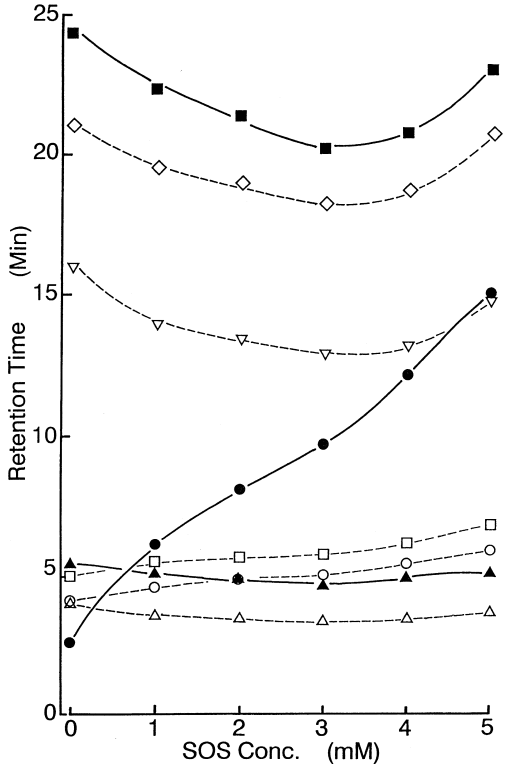

Fig. 9. Effect of sodium 1-octanesulfonate (SOS) concentrations in the HPLC mobile phase on the retention time of 5HT (●), NAS (▲), Mel (■), Trp (○), HIAA (△), MTrp (□), MIAA (▽) and MTPL (◇) [41]. Flow-through time was 1.59 min. The HPLC conditions and the composition of the mobile phase (except for SOS) are the described in Table 1. The applied potential was 900 mV.

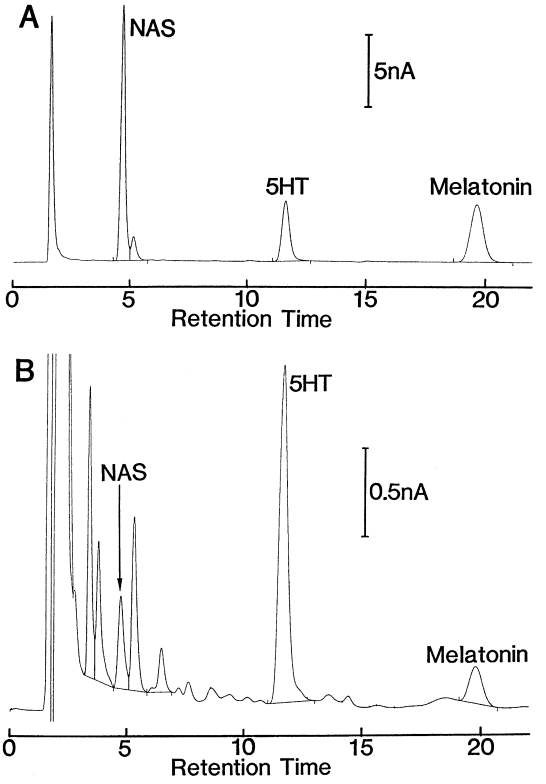

Fig. 10. Simultaneous determination of 5HT, NAS and Mel on one chromatogram by HPLC–ED [41]. (A) Chromatogram of indoleamine standards; 1 ng of each standard was injected; (B) chromatogram of a pineal gland extracted from a juvenile golden hamster at night. The HPLC conditions are described in Table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

extraction steps are required to eliminate non-specific interference in the sample materials. 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. [56] reported that RP-HPLC with FD of plasma without prior extraction can be useful for bioavailability studies of Mel after intravenous and intranasal administration.

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 [59].

6. Conclusion

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

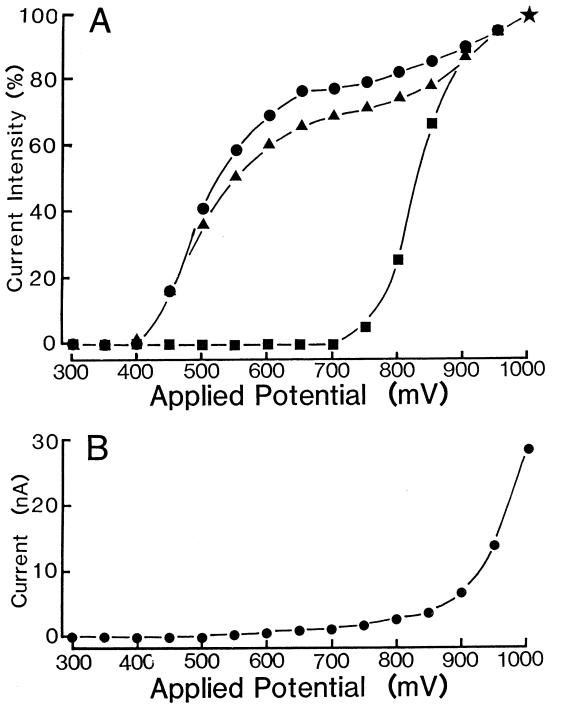

Fig. 11. (A) Hydrodynamic voltammogram for 5HT (●), NAS (▲) and Mel (■). Each point shows the percentage of the current relative to that at 1000 mV of applied potential (asterisk). (B) Background current at each applied potential [41]. The HPLC conditions are described in Table 1. The applied potential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

the accurate quantitative determination of indoleamines by GC–MS. Therefore, HPLC with either FD or ED is better for the routine analysis of indoleamines. Which is the better detection method for pineal indoleamine analysis, FD or ED? Anderson et al. [32] reported that the detection limit of Mel by FD is 25 pg while the limit by ED is 50 pg. Chin [36] also reported that the detection limit of Mel by FD is 60 pg, and the limit by ED is 135 pg. Iinuma et al. [60] 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. On the other hand, the detection limit by ED is reported to be 20 pg [38], 8 pg [43], 5 pg [38,41], and 4 pg [44] without derivatization, whereas the limit by FD was reported to be 35 pg [35] and 10 pg [37]. Therefore, in the routine analyses of Mel without derivatization, ED seems to be more sensitive than FD.

One disadvantage of the use of ED is that the high 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 ED is usually held at less than 30% (v/v). On the other hand, in FD, there is no limit for the concentration of organic solvents. This is a better point in the use of FD because increasing of the concentrations of methanol or acetonitrile in the mobile phase results in shortening of the retention time of Mel. In relation to this, Kulczykowska and Iuvone [57] reported the case of plasma Mel analysis by HPLC with FD using the mobile phase containing 60% methanol. In addition, ED is less sensitive for the Trp than FD [32]. An additional advantage of the use of FD is that the materials detected by FD could be retrieved after detection. It is not possible with ED since ED is a destructive examination.

Although RP-HPLC and GC–MS have been widely used for the separation and determination of pineal indoleamines, other techniques such as high-performance thin-layer chromatography, liquid chromatography–mass spectrometry, micellar electrokinetic chromatography, and capillary electrophoresis (CE) will be applicable in future. There are several papers described the application of CE technique to analysis of 5HT in brain microdialysates [61], 5HT released from mast cells [62], and authentic hydroxyindoles [63]. However, at present, no successful determination has been reported on the pineal indoleamines including Mel using the above mentioned techniques including CE.

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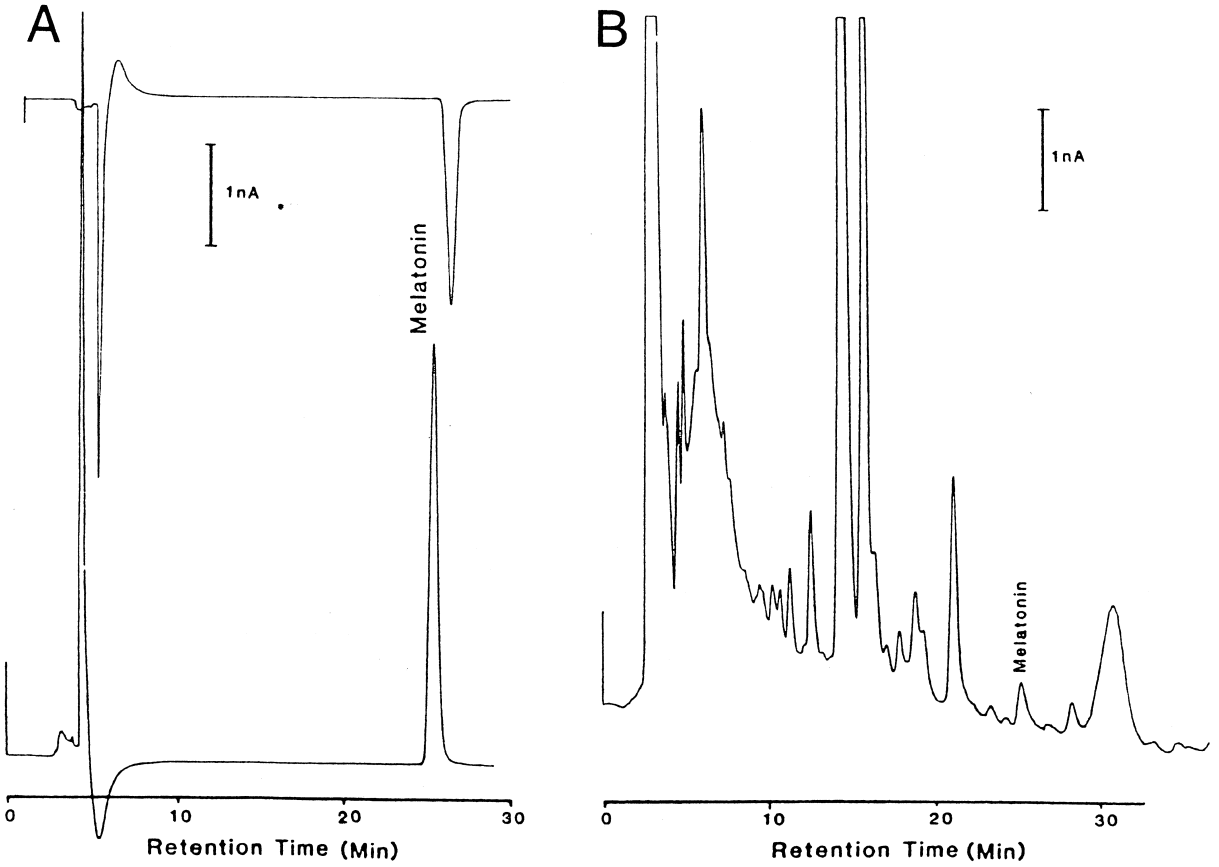

Fig. 12. Determination of plasma Mel by HPLC–ED [58]. (A) Chromatograms of 500 pg of Mel standard and (B) a sample of blood plasma (1.6 ml equivalent) obtained at midnight. The HPLC conditions are described in Table 1. The applied potential was 900 mV.

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