ELSEVIER

Journal of Chromatography B, 774 (2002) 17–24

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of free and total (free plus protein-bound) melatonin in plasma and cerebrospinal fluid by high-performance liquid chromatography with fluorescence detection

Vittoria Rizzo[a,*], Camillo Porta[b], Mauro Moroni[c], Enrico Scoglio[d], Remigio Moratti[a]

[a]*Dipartimento di Biochimica, Sezione Analisi Chimico-Cliniche, IRRCS Policlinico San Matteo, Università degli Studi di Pavia, 27100 Pavia, Italy*

[b]*Medicina Interna e Oncologia Medica, IRRCS Policlinico San Matteo, Università degli Studi di Pavia, 27100 Pavia, Italy*

[c]*Divisione di Oncologia Medica "Falck", Ospedale Niguarda Cà Granda, Milan, Italy*

[d]*ESSECI Group srl, Milan, Italy*

Received 20 July 2001; received in revised form 14 March 2002; accepted 20 March 2002

Abstract

A simple, sensitive and accurate method for the estimation of free and total (free plus protein-bound) melatonin (MLT) in human plasma and cerebrospinal fluid (CSF) is described. Via Chem-Elut cartridges, free and total MLT (the latter obtained after a deproteinization step) were quantified in dichloromethane-extracted samples and analyzed in one chromatographic run by high-performance liquid chromatography (HPLC) with fluorimetric detection. The column used was an Extrasil ODS-2 (3 μm, 150×4.6 mm I.D.), while the mobile phase consisted of 75 m*M* sodium acetate–acetonitrile (72:28, v/v) (pH 5.0). Repeatability and reproducibility of the method were 3.24 and 9.4%, respectively. The recovery of melatonin from plasma and CSF was 99.9±4.0% for non-deproteinized samples and 93.2±4.8% for deproteinized samples. The detection limit of the assay was 0.5 pg/ml. In human plasma, the mean±SD concentrations in the darkness period were 23.18±7.44 pg/ml for free melatonin and 82.5±36.48 pg/ml for total melatonin, while the lowest concentrations detected during daytime were 2.23±2.22 and 7.40±5.68 pg/ml, respectively. Detection of MLT in CSF was 5.01±2.31 and 28.55±6.95 pg/ml for the free and total fraction, respectively.

Keywords: Melatonin

1. Introduction

Melatonin (MLT), or *N*-acetyl-5-methoxytryptamine, is a methoxyindole synthesized from 5-hydroxytryptamine by *N*-acetylation followed by methylation of the 5-hydroxy group. The enzyme that catalyzes this reaction is hydroxyindole-*O*-methyltransferase, largely confined to the pineal body tissue. The circadian change in the activity of this enzyme reflects rhythmical melatonin secretion into the bloodstream with light–darkness alternation [1–3].

A neuromodulator and neurotransmitter, MLT

*Corresponding author. Tel.: +39-038-2502-316; fax: +39-038-2503-517.

E-mail address: vrizzo@venus.it (V. Rizzo).

1570-0232/02/$ – see front matter
PII: S1570-0232(02)00168-X

plays a major role in the interaction between the neuroendocrine and the immune systems by activating specific receptors involved in the regulation of retinal function, circadian rythm, and reproduction [4–6].

Recently, the pharmacological actions of MLT in oxygen radical pathophysiology have been reported [7–9]. Nevertheless, poor MLT bioavailability (about 33%) [10] after drug administration, together with an imbalance between the free and protein-bound fractions, and an equilibration discrepancy between plasma and cerebrospinal fluid (CSF) [11], determines a poorly defined reference value range in terms of both cutoff and baseline values [12].

A number of radioimmunoassay (RIA) methods has been developed to measure MLT in physiological samples [13–15]. For HPLC quantification, several methods based on different extraction procedures have been described, electrochemical [16–18] and fluorimetric [19,20] being the main detection procedures.

Vieira et al. [20] proposed a solvent extraction procedure with dichloromethane and HPLC analysis with electrochemical detection; applying these conditions, it was possible to detect up to 25 pg/ml of MLT in plasma.

To measure the endogenous MLT concentration, Peniston-Bird et al. [21] proposed a HPLC method with fluorimetric detection after solid- and liquid-phase extraction, attaining a sensitivity of 3 pg/ml.

For the estimation of both bound and free MLT in plasma, the non-extraction RIA and the chloroform-extraction RIA procedures, with detection limits of 4 and 5 pg/ml, respectively, were used by Di et al. [22].

In this paper we propose an alternative method for the determination of MLT in human plasma and CSF by isocratic HPLC with fluorimetric detection. The procedure involves an efficient sample clean-up/concentration step using liquid–liquid extraction on Chem-Elut cartridges. The main features of this method are an improved sensitivity with respect to previously reported methods based on the same technique, and a pre-analytical phase with a high sample throughput. This method was validated and used for the quantification of both free and total (free plus protein-bound) MLT.

2. Experimental

2.1. Chemicals

Melatonin and sodium acetate were obtained from Sigma (St. Louis, MO, USA). Dichloromethane and acetonitrile were purchased from Carlo Erba (Milan, Italy). All other solvents and reagents were of analytical grade. Hydromatrix extraction columns (Chem-Elut 1001, 1.0 ml sample capacity) were obtained from Varian (Harborg City, CA, USA). The stock solution of melatonin standard (1 mg/ml) was prepared in a mixture of methanol–water (1:10, v/v) and stored at −20 °C. Working solutions were prepared daily to the desired concentrations, with water. Owing to protein precipitation, the stock standard was diluted with 10% trichloroacetic acid (TCA) (1:0.3, v/v) to give 0.5–500 pg/ml and stored at −20 °C.

2.2. Sample collection

Human plasma was obtained from blood samples collected from the cubital vein of 10 subjects with normal circadian rhythms at 2/4 h intervals for 24 h. Blood treated with anticoagulant was centrifuged at 2000 *g* for 10 min, then plasma was removed, aliquoted (2 ml) and stored at −20 °C until required. CSF obtained from lumbar puncture of lumbar disk hernia patients ($n=18$) was centrifuged at 8000 *g* for 5 min, aliquoted (2 ml) and stored at −20 °C.

2.3. Extraction of total (free plus protein-bound) melatonin

To assess total melatonin concentration, 300 μl of 60 m*M* TCA was added to 1.0 ml of sample. After 10 min in an ice bath, the mixture was centrifuged (5000 *g*, 10 min) and the pH of the supernatant was adjusted to 7.4 by the addition of 20 μl of 1 *M* NaOH. The aqueous sample was added to the top of disposable Chem-Elut extraction columns, without preconditioning washes, adsorbed and distributed into a thin film over the hydrophilic packing material for 3–5 min. Organic extraction was performed with 4 ml of dichloromethane (twice). The extract was concentrated by evaporating the combined eluants in

a waterbath (37 °C). The sample residue was then reconstituted in 60 μl of mobile phase, and 40 μl of this solution was injected into the chromatograph.

2.4. Extraction of free melatonin

Each plasma and CSF sample (1 ml) was added to the top of the extraction columns without deproteinization. The extraction procedure was the same as described for the total compound. For experiments with spiked samples, pooled plasma and CSF were mixed with an appropriate amount of MLT for 30 min at room temperature, to allow equilibration of the analyte with plasma and CSF protein binding sites.

2.5. Statistical analysis

Data are expressed as mean±standard deviation. MLT plasma levels (both of the free and of the total fraction) were compared at different time points, using one-way ANOVA and post-hoc comparison (LSD test). A probability of $P<0.05$ was considered significant.

2.6. Chromatography

Chromatography was performed with an LC-10AD pump, an RF-551 fluorescence detector and a SIL-10AD autosampler (Shimadzu Italia). MLT was separated using an Extrasil ODS-2 (3 μm, 150×4.6 mm I.D.) column (EsseCi Group, Milan, Italy). The HPLC mobile phase consisted of 75 m*M* sodium acetate–acetonitrile (72:28, v/v) (pH 5.0). The flow-rate was 1.0 ml/min. The fluorimetric detector was set at an excitation wavelength of 275 nm and an emission wavelength of 345 nm. The gain was set at ×16. For MLT peak identification, amperometric detection was performed with an EC 41000 detector (Chromsystem, Munich, Germany) at 0.9 V potential. The gain was set at 0.5 nA. Peak area was used as a measure of detector response. Calibration of the chromatographic system for MLT determination was carried out by the external standard method.

3. Results and discussion

3.1. Optimization of sample pretreatment

Several procedures for the selective extraction of free and total MLT from the plasma and CSF samples were explored; solid-phase extraction (using cartridges with different packing materials) and liquid–liquid extraction (using dichloromethane or chloroform as organic solvents as reported in other studies) showed, in our hands, low precision and accuracy. Moreover, a large dilution factor and the presence of several interfering substances made it difficult to assay MLT, especially at high sensitivities. A liquid–liquid extraction step with dichloromethane, via Chem-Elut cartridges, was found to be a more effective procedure than those previously reported in the literature. For extraction of the free fraction of MLT, plasma and CSF samples were added directly to the top of the extraction column, while 10% TCA was chosen as the most suitable sample protein remover (>99%) [23] for the extraction of the total MLT fraction. Two successive extractions with 4 ml of dichloromethane were sufficient to give excellent recoveries of MLT from the Chem-Elut cartridges. The high surface area of a special synthetic inert diatomaceous earth, incorporated into the cartridges, ensures that organic eluents remain uncontaminated by the aqueous matrix, eliminates emulsion problems and facilitates efficient interactions between the sample and the organic solvent extraction. Good reproducible extraction of the analyte was indeed a key step in the development of our method, since no internal standard was employed.

3.2. Chromatographic procedure optimization

Chromatograms of the extracted blank aqueous sample and aqueous standard of MLT equivalent to 2 pg/ml, under isocratic conditions, are presented in Fig. 1. No interfering peaks with the separation and identification of MLT were observed at the retention time of 5 min. Chromatographic conditions for the determination of MLT were investigated and optimized. This compound was strongly retained on a C_{18} stationary phase column, from which it could be

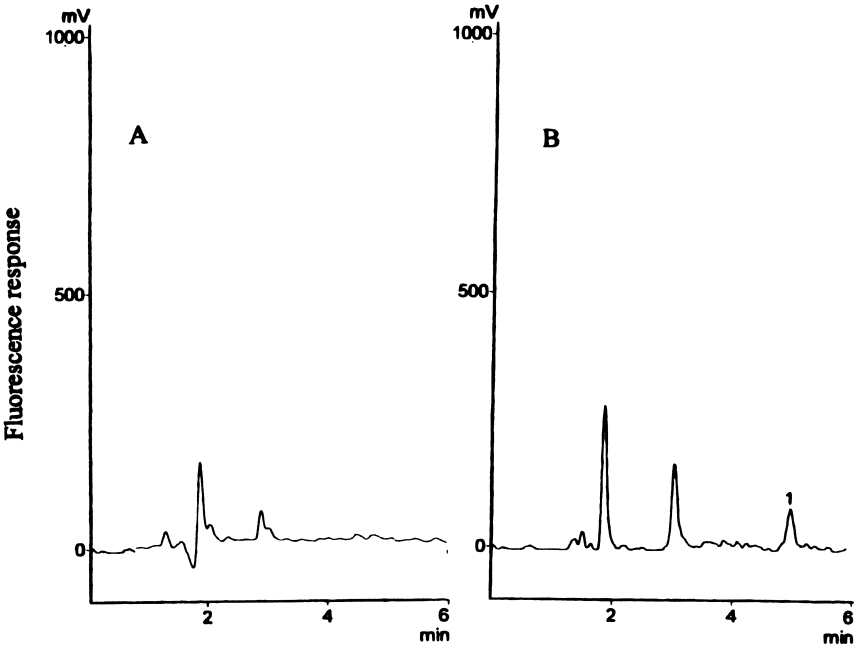

Fig. 1. Chromatograms of extracted blank aqueous sample (A) and aqueous standard of melatonin equivalent to 2 pg/ml (B). For extraction procedure, see Experimental Peak: 1=melatonin.

eluted with mobile phases containing different percentages of organic solvent. We achieved the best result by increasing the percentage of acetonitrile in the mobile phase to 28–30%. A good compromise between peak resolution and feasibility was obtained by appropriate adjustment of the mobile phase ionic strength concentration (acetate buffer, 75 m*M*). The best sensitivity for MLT detection was obtained at pH values between 5.0 and 5.5. As a general-purpose column, we recommend using an Extrasil ODS-2 (3 μm, 150×4.6 mm I.D.) or a column of similar construction. Chromatograms of the extracted MLT free fraction and total fraction in human plasma and cerebrospinal fluid are shown in Fig. 2.

3.3. Quantitative determination

3.3.1. Specificity

Specificity of the isocratic separation was evaluated using fluorimetric and electrochemical detection, in series. The excitation wavelength was evaluated in steps from 270 to 290 nm. Within this range, the fluorescence response of MLT varied less than 40%. Thus, 275 nm was considered satisfactory. The optimum detector potential for MLT amperometric detection was investigated at potentials in the range 0.7–1.20 V. The best signal-to-noise ratio was obtained at 0.9 V. Even though the fluorimetric response is much more sensitive for MLT determination, the chromatograms recorded with fluorimetric and amperometric detection (Fig. 3) show that each peak on the fluorescence plot joins a peak on the electrochemical plot. In this study, the ratio of the fluorescent to the electrochemical signal was used as a tool for a positive identification of MLT in a single chromatographic run. A good correlation of the fluorimetric/electrochemical ratio (1.17±0.018, C.V. 1.53%) was obtained for standard solutions and plasma and CSF samples, indicating a pure MLT extraction without analyte contamination.

3.3.2. Linearity

Calibration curves ($n=5$) of a wide range of MLT aqueous standards in concentrations varying from 0.5 to 500 pg/ml, extracted as described, were determined. Calibration curves ($n=5$) were also determined for pooled plasma and CSF samples spiked with known amounts of MLT, before extraction. The

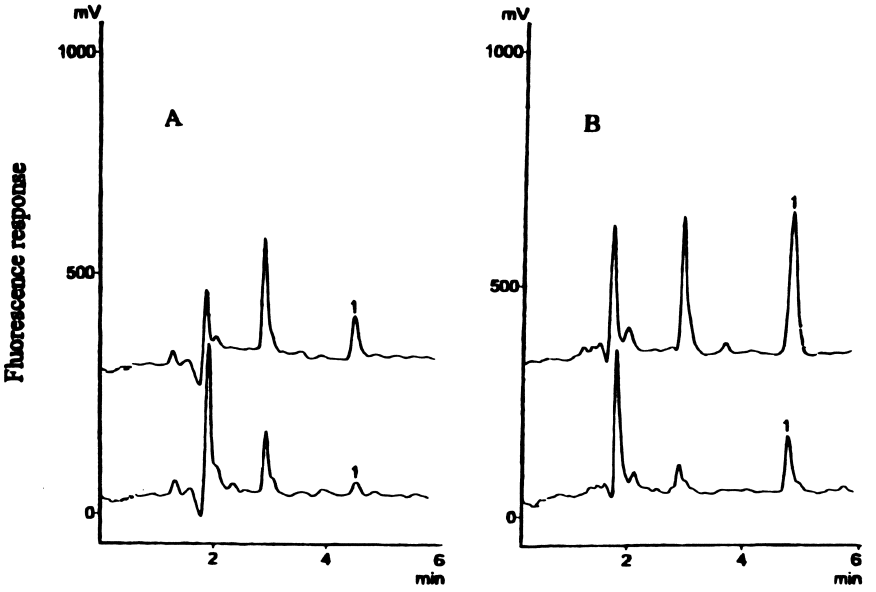

Fig. 2. Chromatograms of extracted melatonin free fraction (lower trace) and total fraction (upper trace) in plasma (A) and cerebrospinal fluid (B). Plasma melatonin free and total concentrations were 1.1 and 3.7 pg/ml, respectively. CSF melatonin free and total concentrations were 5.3 and 28 pg/ml, respectively. Column, Extrasil ODS-2 (3 μm, 150×4.6 mm I.D.); mobile phase, 75 m*M* sodium acetate–acetonitrile (72:28, v/v); pH 5.0; flow-rate, 1.0 ml/min. Fluorimetric detection: $\lambda_{ex} = 275$ nm, $\lambda_{em} = 345$ nm. Peak: 1 = melatonin.

linear correlation was found to be $y = 0.84x - 0.31$ ($r^2 = 0.9996$) for MLT aqueous standard extracts, $y = 0.89x + 0.23$ ($r^2 = 0.9991$) for MLT in pooled plasma sample extracts and $y = 0.84x + 0.04$ ($r^2 = 0.9993$) in pooled CSF extracts.

3.3.3. Recovery

The extraction recovery of MLT from the undeproteinized and deproteinized pooled plasma and CSF samples was determined by spiking known amounts of MLT (0.5, 5.0, 50, 500 pg/ml), and was calculated by comparing the peak response with those obtained from MLT aqueous standard extracts (0.5, 5.0, 50, 500 pg/ml). It was found that the mean±SD of the recovery was 99.9±4.0% for undeproteinized samples and 93.2±4.8% for deproteinized samples. The recoveries in percent are shown in Table 1.

3.3.4. Precision

Precision was defined in terms of repeatability and reproducibility. Repeatability (intra-assay variation) was determined in 10 plasma extracted replicate runs at the same concentration (10 pg/ml). Reproducibility (inter-assay variation) was determined in 10 plasma extracted aliquots analyzed on different days at the same concentration (10 pg/ml). Precision data with a C.V. of 3.24 and 9.4%, respectively, fall within accepted values for clinical analyses.

3.3.5. Limit of detection and quantitation

The detection limit (LOD), based on a signal-to-noise ratio of 3:1, was 10±0.5 pg/ml, 2.67% C.V., for MLT unextracted aqueous standard. This value, considering a 20-fold concentration factor for MLT extracted from aqueous standards, plasma and CSF, corresponds to 0.5 pg/ml, the minimum quantitation

Table 1
Recoveries of MLT of various concentrations in plasma and CSF

MLT conc. (pg/ml)	Non-deproteinized samples		Deproteinized samples	
	Recovery (%) (mean±SD)	C.V. (%)	Recovery (%) (mean±SD)	C.V. (%)
0.5	96.9±2.02	2.0	92.2±4.65	5.0
5.0	101.4±4.56	4.0	89.9±4.87	5.0
50	104.0±4.52	4.5	92.8±4.76	5.0
500	97.8±1.92	2.0	94.0±5.56	6.0

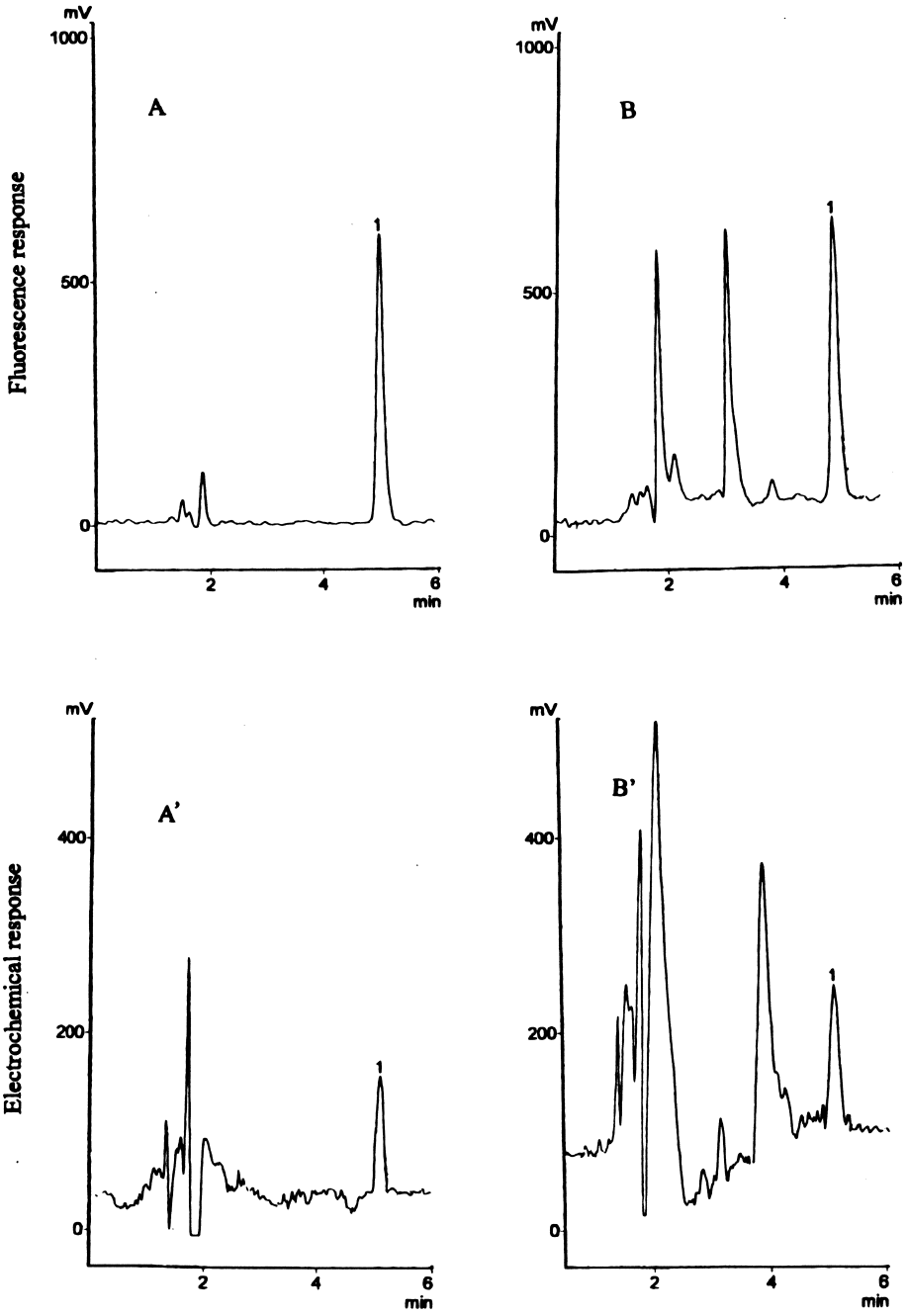

Fig. 3. Peak melatonin identification by fluorimetric and electrochemical detection, in series. The fluorescent response of melatonin in extracted aqueous standard (A) and in the CSF sample (B) together with the electrochemical response in both extracted aqueous standard solution (A′) and in the CSF sample (B′). Extracted melatonin aqueous standard concentration: 30 pg/ml. CSF sample melatonin concentration: 28 pg/ml. Fluorimetric detection: $\lambda_{ex} = 275$ nm, $\lambda_{em} = 345$ nm.. Amperometric detection: 0.90 V. Peak: 1 = melatonin.

Table 2
Twenty-four hour profile of free and total (free plus protein-bound) fractions of circulating melatonin in plasma

	Evening from 20:00 to 24:00 h	Night from 02:00 to 04:00 h	Day from 08:00 to 16:00 h
Melatonin free fraction	9.31±3.37* (5.0–16.5)	23.18±7.44* (14.2–38.0)	2.23±2.22* (0.5–7.9)
Melatonin total (free plus protein-bound) fraction	32.2±21.1* (8.0–82.0)	82.5±36.48* (41.0–163)	7.40±5.68* (0.9–17.0)

Values are expressed as mean (pg/ml)±standard deviation. Ranges (pg/ml) are in parentheses. Number of samples 10.
*$P<0.0001$, ANOVA. Differences observed at the three given time points were all statistically significant.

(LOQ) that could be measured routinely with less than 3% repeatability.

3.3.6. Quantitative analysis of samples

The described method was used to determine MLT free and total levels in plasma and CSF samples. The mean concentration of circulating MLT free fraction was 10.12±9.30 pg/ml, which corresponds to 28.54% of the total fraction of 35.45±36.7 pg/ml in plasma samples collected from healthy subjects. The significant changes in plasma MLT levels determined at three time points ($P<0.0001$, ANOVA) indicates a characteristic circadian profile (Table 2).

In the evening, the MLT level was 9.31±3.37 pg/ml for the free fraction and 32.2±21.1 pg/ml for the total fraction. Higher MLT levels of 23.18±7.44 pg/ml for the free fraction and 82.5±36.48 pg/ml for the total fraction were obtained in the middle of the night, while the lowest concentration (2.23±2.22 pg/ml for the free fraction and 7.40±5.68 pg/ml for the total fraction) was detected during the day.

MLT levels in CSF samples were similar to or lower than those found in plasma, corresponding to 5.01±2.31 pg/ml for the free fraction and 28.55±6.95 pg/ml for the total fraction. Moreover, the observation that the protein-bound fraction was between 69.9 and 71.1% at any given plasma MLT concentration and between 82.5 and 87.4% in CSF samples indicates that MLT binds to plasma and CSF proteins to a major extent and the free fraction does not appear to be influenced by the total concentration present in the sample.

These results suggest that the estimation of free and total MLT in CSF and plasma should be investigated independently, as suggested in previous studies [24]. Thus, quantification of both free and total MLT could provide important information, especially when the variation in plasma and CSF protein binding is associated with various pathological conditions.

4. Conclusions

The method described was developed specifically for the determination of free and total MLT in human plasma and CSF. The assay is reproducible, can be performed without an internal standard, and can detect concentrations as low as 0.5 pg/ml with only 1.0 ml of sample. Other advantages include a short retention time, a short run time, simple sample preparation, and a simple extraction procedure with good recovery of MLT. We conclude that this HPLC method is reliable and its simplicity will make it valuable for both biochemical and clinical use.

References

[1] A. Brzezinski, New Engl. J. Med. 16 (1997) 186.
[2] J. Axelrod, H. Weissbach, Science 131 (1960) 1312.
[3] C.M. Craft, in: H.S. Yu, R.J. Reiter (Eds.), Melatonin: Biosynthesis, Physiological Effects and Clinical Applications, CRC Press, Boca Raton, FL, 1992, p. 17.
[4] F. Waldhauser, M. Waldhauser, H.R. Lieberman, M.H. Deng, H.J. Linch, R.J. Wurtman, Neuroendocrinology 39 (1984) 307.
[5] S.L. Berga, S.S. Yen, Neuroendocrinology 51 (1990) 606.
[6] R.C. Zimmerman, S. Schroder, S. Baars, M. Schumacher, H.C. Weise, Fertil. Steril. 54 (1990) 612.
[7] R. Reiter, L. Tang, J.J. Garcia, A. Munoz-Hoyos, Life Sci. 60 (1997) 2255.

[8] P. Lissoni, S. Barni, S. Meregalli, V. Fossati, M. Cazzaniga, D. Esposti, G. Tancini, Br. J. Cancer 71 (1995) 854.
[9] P. Lissoni, S. Meregalli, L. Nosetto, S. Barni, G. Tancini, V. Fossati, G. Maestroni, Oncology 53 (1996) 43.
[10] W.-L. Di, A. Kadua, A. Johnston, R. Silman, New Engl. J. Med. 3 (1997) 1028.
[11] D. Le Bars, P. Thivolle, P.A. Vitte, C. Bojkowski, G. Chazot, J. Arendt, Int. J. Rad. Appl. Instr. B 18 (1991) 357.
[12] G. Hajak, A. Odenbeck, J. Staed, B. Bandelow, G. Huether, E. Ruther, J. Pineal Res. 19 (1995) 116.
[13] J. Arendt, L. Wetterberg, T. Heyden, P.C. Sizonenko, L. Paunier, Horm. Res. 8 (1977) 65.
[14] J.F. Peniston-Bird, W.L. Di, C.A. Street, R. Edwards, J.A. Little, R.E. Silman, J. Pineal Res. 20 (1996) 51.
[15] B. Poeggeller, R. Hardeland, J. Pineal Res. 17 (1994) 1.
[16] A. Conti, G.J.M. Maestroni, J. Pineal Res. 20 (1996) 138.
[17] F. Raynaud, P. Pevet, J. Chromatogr. 564 (1991) 103.
[18] W.J. Drijfhout, C.J. Grol, B.H. Westerink, J. Neurochem. 61 (1993) 936.
[19] G.F. Oxenkrug, P.J. Requintina, Int. J. Neurosci. 77 (1994) 237.
[20] R. Vieira, J. Miguez, M. Lema, M. Aldegunde, Anal. Biochem. 205 (1992) 300.
[21] J.F. Peniston-Bird, W.-L. Di, C.A. Street, A. Kadva, M.A. Stalteri, R.E. Silman, Clin. Chem. 39 (1993) 2242.
[22] W.-L. Di, A. Kadua, A. Djahanbakhch, R. Silman, Clin. Chem. 44 (1998) 304.
[23] V. Rizzo, A. Anesi, L. Montalbetti, G. Bellantoni, R. Trotti, G.V. Melzi d'Eril, J. Chromatogr. A 729 (1996) 181.
[24] D.P. Cardinali, H.J. Lynch, R.J. Wurtman, Endocrinology 91 (1972) 1213.