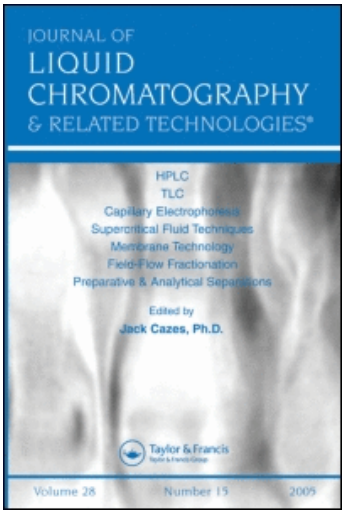

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DETERMINATION OF MELATONIN IN COMMERCIALLY AVAILABLE PRODUCTS BY LCEC AND LC/MS/MS

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ABSTRACT

Simple and reliable methods for liquid chromatography/electrochemistry (LCEC) and LC/MS/MS with atmospheric pressure chemical ionization (APCI) have been developed for the determination of melatonin in commercially available products. The dissolution of melatonin from the melatonin-containing formulations was simply obtained by dissolving the tablets and capsules in 20% acetonitrile in 0.1 N perchloric acid with ultrasonication. The filtrate of the resultant solution was separated on a 1 x 150 mm C_{18} microbore column for LCEC, and also on a 3.2 x 100 mm C_{18} column for ion trap LC/MS/MS. The linear response ranges for melatonin were 0.1 ng - 1 ng on column using LCEC and 0.5 ng - 5 ng on column using LC/MS/MS.

The agreement between LCEC and LC/MS/MS is very good and the correlation coefficient between these two methods for the analysis of 40 sets of samples prepared from 5 kinds of melatonin-containing products is 0.998.

INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine, MLTN) is well known as one of the neurochemically important indole analogues, which is primarily biosynthesized from tryptophan in the pineal gland and controls circadian rhythms.[1-3] It has been used as a nonregulated therapeutic agent against jet-lag[4] and sleep disorders.[5] Recent work demonstrates that, even at a very low dosage regimen (0.3 mg per day), MLTN can rapidly and extensively induce sleep in patients suffering from insomnia.[6] For these reasons, there are many commercially available melatonin-containing products with a range of potency (0.2 mg - 3 mg). Due to the unregulated nature of melatonin in the USA, there have been questions raised about the quality of products. Simple and reliable methods for monitoring MLTN and possible precursors and degradation products are desirable to establish potency and stability. MLTN in biological samples has been determined by radioimmunoassay, GC/MS, and LCEC and/or LC with fluorescence detection,[6-10] for situations where the MLTN concentration is extremely low (often pg/mL). In this study, we describe LCEC and LC/MS/MS methods for the determination of MLTN in commercially available products. The two methods were bench-marked against each other providing a high degree of confidence in both.

MATERIALS AND METHODS

Chemicals and Standards

MLTN (99.9% purity, HPLC grade), L-tryptophan (TPN), 5-hydroxy-indoleacetic acid (5-HIAA), serotonin (5-HT), and 5-hydroxy-L-tryptophan (5-OH TPN) were obtained from Research Biochemicals International, Natick, MA, USA. 5-Methoxy-indoleacetic acid (5-MIAA), N-acetyl-5-hydroxy-tryptamine (NAcHT), 5-methoxytryptophol (5-M TOL), N-methyl-5-hydroxytryptamine (NMHT), 5-hydroxytryptophol (5-OH TOL), and 5-methoxytryptamine (5-MT) were purchased from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals used for LCEC and LC/MS/MS analysis were of analytical-reagent grade and used as received. Commercial products of melatonin-containing tablets and capsules were purchased from local sources.

Preparation of Tablets and Capsules

Eight tablets or capsules from the same lot of each product were randomly taken and separately placed in 100 mL volumetric flasks. Sample preparation solution (20% acetonitrile with 0.1 M perchloric acid) was added to each flask to volume, and the flasks were sonicated in a water bath (at ambient temperature) for approximately 10 to 30 minutes to ensure complete dissolution of MLTN from its excipients. The resultant solution was filtered through a 0.2 μm membrane microfilter (MF-1 centrifugal microfilter, BAS, West Lafayette, IN 47906, USA). Each filtrate was diluted with distilled water to the apparent concentration (based on the MLTN label value) of 100 pg/μL and 1 ng/μL and then subjected to LCEC and LC/MS/MS analysis, respectively.

The effect of changing the composition of the sample preparation solution, its volume, and sonication time, and effects of different sample preparation solvents on the stability of MLTN were investigated. It was found that the sample preparation solvent and procedure described above provided complete recovery of MLTN and stable samples for the determination of MLTN in its formulations.

LCEC

Determination of MLTN by LCEC was performed on a BAS 200B chromatograph equipped with a low dead volume micro-injector (5 μL loop, BAS). The analytical column was a UniJet C_{18} 5 μm, 150 mm x 1 mm I.D. (BAS). A UniJet C_{18} column, 5 μm, 100 mm x 1 mm I.D. (BAS) was installed before the injector to raise the overall system pressure for optimal pump performance. The oven temperature containing the columns and detector was maintained at 35°C. The mobile phase was 20% acetonitrile with buffer solution of 15 mM sodium perchlorate, 40.6 mM sodium citrate, 2.15 mM sodium octylsulfonate, 10 mM diethylamine hydrochloride, and 27 μM disodium EDTA. The flow rate was 100 μL/min. Electrochemical detection with a glassy carbon working electrode (3 mm) was at an applied potential +850 mV vs. Ag/AgCl.

LC/MS/MS

The LC/MS/MS was equipped with a BAS PM-80 pump (BAS) coupled to a Finnigan LCQ ion trap mass spectrometer with a built-in injector (5 μL loop) (Finningan MAT, San Jose, CA, USA). The separation of MLTN was carried out on a Biophase II C_{18} column, 3 μm, 100 mm x 3.2 mm I.D. (BAS).

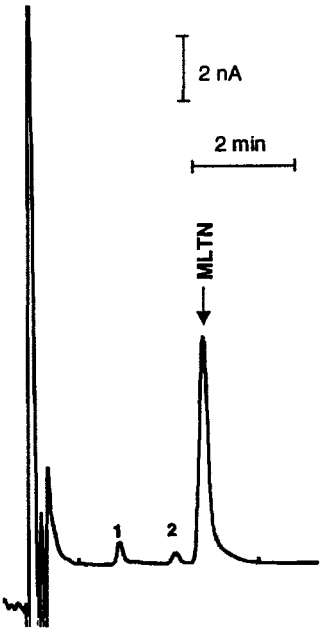


Figure 1. Chromatogram of melatonin in tablet using LCEC. Peaks: 1 = unknown additive in tablet; 2 = system peak.

The mobile phase was 20 % acetonitrile and 1 % acetic acid in 10 mM ammonium acetate. The flow rate was 1 mL/min. NAcHT (1 ng/μL) was used as an internal standard (IS) for the determination of MLTN. The LC effluent was pumped into the APCI source using a vaporizer temperature of 450°C and a capillary temperature of 150°C. MLTN and IS were monitored using the selected reaction monitoring (SRM) mode. The parent ions of IS m/z 219 and MLTN m/z 233 were mass-selected while the product ions of m/z 160 and m/z 174 were monitored with a 1 amu mass window for IS and MLTN, respectively. All spectra were in the positive ion mode, positive chemical ionization was effected by a corona discharge needle (+4 μA). The flow rates of the sheath gas and auxiliary gas were set at 70 and 2 units, respectively. Two segments of a scan event were used. The first segment (m/z 219 → 160) was used to monitor the IS for the first 1.5 minutes while the second segment (m/z 233 → 174) was used to monitor MLTN for the next 2.5 minutes. Collision-induced dissociation of the parent ions with helium gas was performed at 17% collision energy (i.e., 0.85 Vp-p applied to the end-caps).

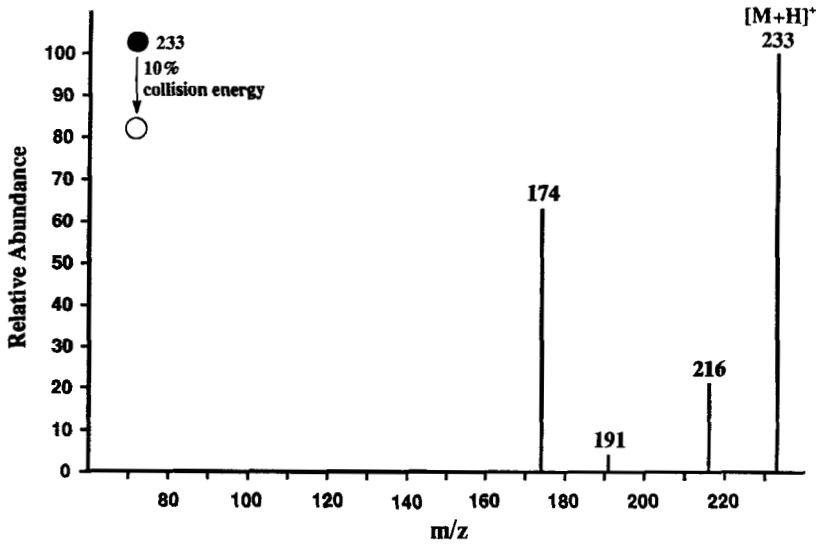
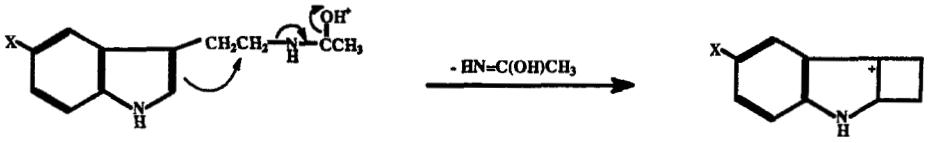


Figure 2. Mass spectrum of the collision induced dissociation of the protonated melatonin (m/z 233).



Scheme 1. Mechanism of the fragmentation of the protonated melatonin and the internal standard.

RESULTS AND DISCUSSION

Typical chromatogram of sample from commercial melatonin-containing tablet obtained by using LCEC is shown in Figure 1. MLTN was detected at 4.13 minutes and there is no evident interfering peak in any of the formulations to be found on chromatograms.

For LC/MS/MS, collision induced dissociation of the protonated melatonin m/z 233 yields predominately the fragment ion at m/z 174 as shown in Figure 2. A possible mechanism is shown in Scheme 1.

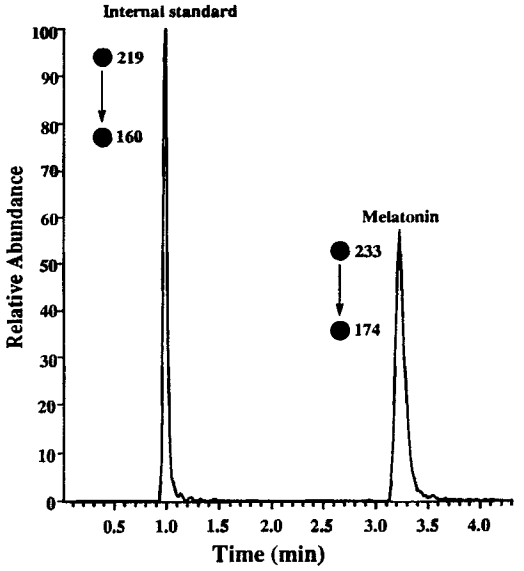


Figure 3. Chromatogram of NAcHT (IS) and melatonin using LC/MS/MS.

Since monitoring the product ions of m/z 160 and m/z 174 produced from their parent ions of m/z 219 and m/z 233 are more selective for the determination of IS and MLTN, respectively, these ions were then selected for the SRM (Scheme 1). The retention times of IS and MLTN are 0.97 minutes and 3.21 minutes, respectively. A typical chromatogram obtained by using LC/MS/MS is shown in Figure 3. In both the LCEC and LC/MS/MS case the chromatograms are clean and reproducible.

The calibration curve for MLTN was linear over the range of 0.1 ng - 1 ng on column for LCEC by plotting the peak area of MLTN against its amount, and of 0.5 ng - 5 ng for LC/MS/MS by plotting the ratio of peak area of MLTN to IS against the amount of MLTN. Linear regression parameters (Table 1) measured by these two methods show good linearity.

The injected detection limits for MLTN, calculated as a signal-to-noise ratio of 3:1, are 3 pg on column by using LCEC and 50 pg on column by using LC/MS/MS. The sensitivity of both methods is more than sufficient to detect and quantitate MLTN in commercial melatonin-containing products.

Table 1

Linear Regression Data for Melatonin Calibration Curve Obtained by Using LCEC and LC/MS/MS

Parameter[a]	Methods	
	LCEC	LC/MS/MS
y	$-2.24677/10^3 + 6.0690/10^3$ x	0.017024 + 1.063106 x
r	0.9999	0.9984
r^2	0.9998	0.9968

[a] y = peak area, x = amount of MLTN, r = coefficient of correlation.

Evaluation of effects of temperature on the stability of MLTN in the prepared samples of the commercially available melatonin-containing products was carried out by the determination of MLTN in prepared samples stored at room temperature, in refrigerator and at -20°C. It was found that MLTN in prepared samples is stable for at least two days at room temperature, one week stored in refrigerator, and one month stored at - 20°C for all of the tested formulations. When the prepared samples were stored in a refrigerator for more than one month, the amounts of MLTN decreased. We checked all of the in-hand standards of indole analogues, such as 5-HT, TPN, 5-HIAA, 5-OH TPN, 5-MIAA, NAcHT, 5-M TOL, NMHT, 5-OH TOL, and 5-MT, by LCEC and LC/MS/MS; however, none of them was found to be the decomposition substance. More detailed studies on the decomposition of MLTN are being conducted in this laboratory and the results will be reported.

Four brands of tablets, products A, B, C, E, claiming the content of MLTN of 0.2, 0.5, 1, 3 mg per tablet and one brand of capsule, product D, claiming the content of MLTN of 2.5 mg per capsule were analyzed by using the developed methods. As shown in Table 2, the variations of the content of MLTN among products A, B, and E were 4.1 % to 6.7 %, it showed the content uniformity of MLTN in these products is acceptable. The variations of the content of MLTN in product C and product D are 30 % and 11 %, which are rather poor.

The MLTN content varied from a lower value of 80 % of the claim amount to a higher value of 110 % of the claim amount in the different formulations from different manufacturers. Products D and E, 99.6 % to 106 % of the claim content of MLTN were found. Only 80 % - 85 % and 90 % - 92 %

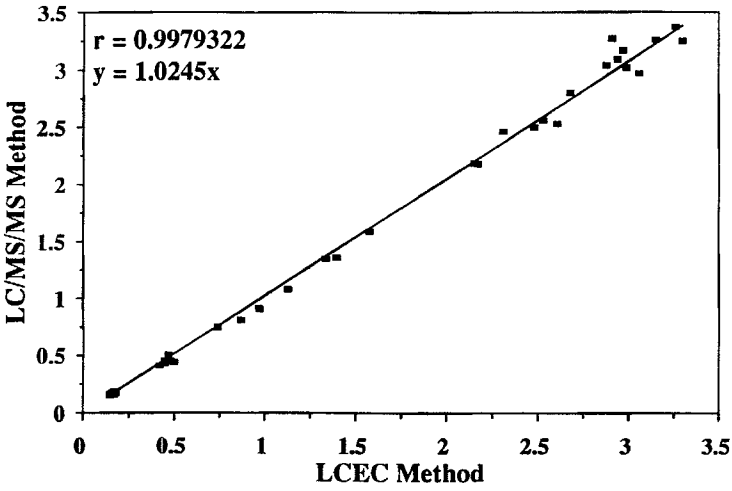


Figure 4. Correlation between LCEC and LC/MS/MS for the determination of melatonin in the melatonin-containing products.

Table 2

Content of Melatonin Found and Claimed in Commercial Products

Prod.	A		B		C		D		E	
	LC/MS /MS	LCEC	LC/MS /MS	LCEC	LC/MS /MS	LCEC	LC/MS /MS	LCEC	LC/MS /MS	LCEC
mg of	0.18	0.17	0.44	0.50	1.36	1.40	2.46	2.31	3.27	2.91
melatonin	0.16	0.17	0.41	0.42	1.59	1.58	2.80	2.68	3.37	3.26
per tablet	0.17	0.17	0.45	0.45	1.35	1.34	2.53	2.61	3.17	2.97
or capsule	0.17	0.18	0.45	0.48	0.75	0.74	2.50	2.48	3.26	3.15
found	0.16	0.15	0.45	0.45	0.74	0.74	2.56	2.53	2.97	3.06
	0.17	0.16	0.43	0.45	1.08	1.13	2.18	2.17	3.04	2.88
	0.15	0.15	0.48	0.47	0.91	0.97	2.19	2.15	3.09	2.94
	0.16	0.15	0.50	0.47	0.81	0.87	3.02	2.99	3.25	3.30
Mean	0.17	0.16	0.45	0.46	1.07	1.10	2.53	2.49	3.18	3.06
S.D.	0.01	0.01	0.03	0.02	0.32	0.32	0.28	0.28	0.13	0.16
R.S.D. (%)	5.9	6.3	6.7	4.3	30	29	11	11	4.1	5.2
Claim Content	0.2		0.5		1		2.5		3	
% Claim Content	85	80	90	92	107	110	101	99.6	106	102

of the label claim were found in products A and B, respectively, while 107 % - 110 % of the label claim were found in product C. These results demonstrate that the developed methods are useful for the quality control of melatonin-containing products.

The agreement between the LCEC and LC/MS/MS for MLTN analysis was investigated by simultaneously analyzing 40 sets of samples prepared from 5 kinds of melatonin-containing products using these two methods. Results in Table 1 show comparable data obtained by using these methods. Linear regression analysis gave a coefficient (r) of correlation of 0.998 and an equation of the regression line $y = 1.0245\ x$ (Figure 4). These results suggest that the developed methods compare very well.

CONCLUSION

The present study has demonstrated that LCEC and LC/MS/MS provided satisfactory methods for determination of MLTN in melatonin-containing products. The sample preparation procedure is simple and reliable. LCEC is less costly, while LC/MS/MS can provide both quantitation and confident identification of drug and impurities simultaneously.

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