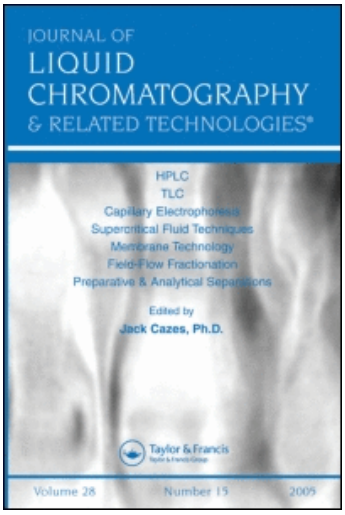

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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M. Hariharan[a]; J. S. Naickar[ab]; A. S. Hilibrand[a]
[a] Departments of Psychiatry, University of Michigan Medical School, Michigan Michigan [b] Orthopedic Surgery, Michigan

To cite this Article Hariharan, M. , Naickar, J. S. and Hilibrand, A. S.(1996) 'Melatonin Determination in Human Urine by Liquid Chromatography Using Flurometric Detection', Journal of Liquid Chromatography & Related Technologies, 19: 11, 1745 — 1756
To link to this Article: DOI: 10.1080/10826079608014002
URL: http://dx.doi.org/10.1080/10826079608014002

J. LIQ. CHROM. & REL. TECHNOL., 19(11), 1745–1756 (1996)

MELATONIN DETERMINATION IN HUMAN URINE BY LIQUID CHROMATOGRAPHY USING FLUROMETRIC DETECTION

M. Hariharan, Janaki S. Naickar,* A. S. Hilibrand

University of Michigan Medical School
Departments of Psychiatry and
*Orthopedic Surgery
1150 W. Medical Center Dr., MSRB-1
Ann Arbor, Michigan 48109-0656

ABSTRACT

A new, simple, very sensitive, highly reproducible, isocratic, and reverse phase liquid chromatographic method has been developed for determining urine melatonin concentrations using a fluorometric detector. 6-methoxymelatonin is used as an internal standard. A C_{18} column (150 X 0.46 cm) and a "Waters" fluorometric detector (Model 474 with a 16 uL flow cell are the main components of the liquid chromatographic system. The excitation and emission wavelengths are 285 and 335 nm respectively. Melatonin is extracted from urine at pH 7 using chloroform. The method is sensitive to 0.5 pg. The mean (n=6) inter-assay precision (CV) is 4.3% and intra-assay precision is 5.8%. The mean absolute recovery of the method is about 90%.

INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine) is a hormone of pineal origin. The circadian function of the pineal gland is reflected by the diurnal variation in the levels of melatonin in the blood. The highest level is noted in the night and the abnormal levels of the hormone are implicated in reproductive malfunctions and neurological and psychiatric disorders.[1] The extensive literature regarding the physiological and endocrinological role of melatonin[2] reveals the high level interest in the hormone.

There are many published methods for the analysis of melatonin in biological samples, including the radioimmunoassay (RIA),[3] gas chromatography-mass spectrometry,[4] and liquid chromatography.[5-9] The high performance liquid and chromatographic (HPLC) methods using fluorometric and electrochemical detectors are simple, cost effective, specific and seem to be more commonly used than the less sensitive RIA method. Most of the published HPLC methods do not use an internal standard, except one HPLC-UV method.[10] The utilization of an internal standard in an HPLC method involving multiple extraction and wash steps provides distinct advantages and enhances its quality. The use of 6-methoxy melatonin as an internal standard in the assay is a very good choice, because of its close structural similarity to melatonin, and no one has used the compound in the assay.

Careful study of the different HPLC analytical methods for melatonin reveals that the present methods totally lack the comprehensive features necessary for sensitive work. Our, new and simple, assay has been used to test clinically the validity of a hypothesis based on animal experiments, that idiopathic scoliosis may be reflected by the pineal gland function in adolescence.[11,12]

A total of 80 day time and night time, spot urine samples from both controls and patients were analyzed by the new HPLC method.

MATERIALS AND METHODS

Melatonin and other compounds used in interference studies in the method were obtained from Sigma, St. Louis, MO, USA; 6-methoxy melatonin was from Research Biochemicals, Inc., Natick, MA, USA. Monobasic potassium phosphate and triethylamine, and reagent grade chloroform, HPLC grade solvents acetonitrile and methanol were from Mallinkrodt Chemicals Co., Paris, KY, USA. All extractions were done in 25 mL all Teflon screw capped tubes (Nalgene) from VWR Scientific, Chicago, IL.

The HPLC system consisted of a pump by Hitachi (Danbury, CT, USA, Model L-6000), a C_{18}, 3 μm Econosphere column (150X 4.6 mm, Alltech Associates, Dearfield, IL, USA), an autosampler, from Waters (Model 717, Milford, MA, USA), a self packed guard column from Upchurch (Oak Harbor, WA, Model C-130-B) containing Perisorb RP-18 material, a fluorometric detector from Waters (Model 474) with a 16 μL flow cell and an integrator from Hitachi (Model D 2500). The excitation and emission wavelengths for the detector in the assay were 285 and 345 mm respectively.

The mobile phase is a mixture of phosphate (KH_2PO_4) buffer (30 mmol, containing 5 mL of triethylamine per litre and its pH adjusted to 4.3) acetonitrile and methanol (85 : 11 : 4 v/v). The flow rate is 1.35 mL/min.

Primary stock standard solutions of melatonin and 6-methoxy melatonin contained 1 mg of compound per mL of methanol solution. Secondary standards were prepared in 0.1% ascorbic acid solution. Aqueous working standards of melatonin containing 25, 62.5, 125, 250 pg/mL solution were prepared freshly ,on assay days, using a ng/mL standard. The internal standard solution (6-methoxymelatonin) contained 50 ng of compound per 50 μL of aqueous solution.

All primary and secondary standards were aliquotted and kept frozen at -80°C. These standards were found to be stable for 6 months.

Patient Samples

The study involved 9 female adolescent patients determined to have scoliosis and 18 healthy female controls of the same age group with no medical problems. All participants provided two spot urine samples, one of them was the evening void corresponding to day time melatonin production, and the second sample was the first morning void corresponding to over-night time melatonin production. All samples were preserved with 1g of boric acid and kept at -80°C until analysis.

Extraction

Pipet 8 mL of urine or aqueous standard or quality control sample into a 25 mL Teflon tube, adjust its pH to 7.0, and add 50 μL (50 ng) of 6-methoxymelatonin and 10 mL of chloroform. Cap the tubes and shake them in a horizontal shaker for 30 min. Centrifuge the tubes for 10 min and aspirate the top aqueous layer. Wash the chloroform extract successively with 8 mL

each of 0.1M sodium hydroxide and water. Agitate the tubes with wash reagent for 10 min, centrifuge and aspirate off the top aqueous layer. To the final chloroform solution add 250 μL of 0.05M methanolic hydrochloric acid, vortex and evaporate the chloroform under nitrogen in a 45°C water bath. Reconstitute the residue in 150 μL of mobile phase and inject 60 μL into the HPLC column.

METHOD DEVELOPMENT

Melatonin concentration in urine is of the order of a few pico grams per mL. A simple, very sensitive, and specific method is required. Both electrochemical[7,9] and fluorometric[5,8] detectors used in liquid chromatographic work, enables one to achieve the low detection limits. Urine is our choice specimen for this work, since, it provides a good mean concentration for melatonin produced both during day and over-night.

There are three advantages in choosing urine over plasma: one, it is easy to obtain, two, large volumes of sample (8-10 mL) can be used for extraction and, three, it reflects the mean concentration of melatonin produced over a period of several hours.[13]

Our attempts to use the electrochemical detector (Coulochem model 5100A by Environmental Science Associates, Bedford, MA, USA) in the work, proved very difficult because of the highly complex nature of the urine matrix and the high voltage setting required for the method. On the other hand, chromatograms of urine extracts obtained using fluorometric detectors are relatively less complex. We readily adjusted the mobile phase composition for our choice of C_{18} column, and fit the internal standard (6-methoxymelatonin) in the chromatogram in a location free of urine matrix interferences.

One of the most important requisites for achieving the low detection limits in this assay, is the use of a sensitive fluorometric detector. We found the Shimadzu fluorometric detector (Model RF 551 with a 12 μL flow cell) to be quite inadequate at the lower end of the detection limits of the assay. The "Waters" scanning fluorometric detector (Model 474 with a 16 μL flow cell), clearly enabled us the detection of less than 1 pg concentration of melatonin.

Direct injection of 0.5 pg of melatonin into the column gave a peak of 2 cm height at an attenuation of 1, and the noise to signal ratio was < 0.025. We found the "Waters" detector to be about forty times more sensitive than the Shimadzu RF 551 detector at its low sensitivity setting.

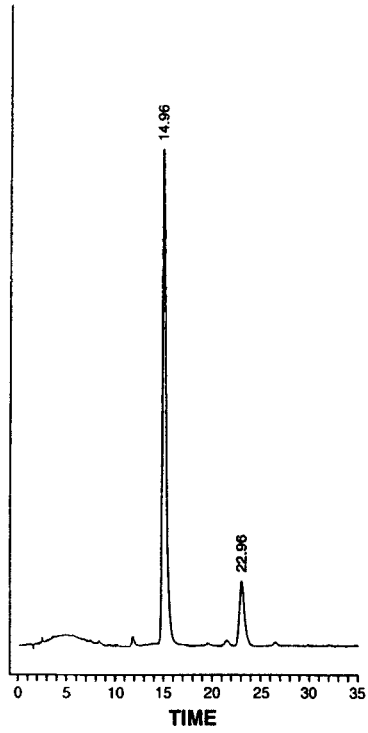

Figure 1. Chromatogram of an aqueous standard extract containing 125 pg/mL of melatonin and 50 ng of 6-methoxy melatonin.

Yet another important factor for the good sensitive nature of this assay method was our pH optimization studies of the extraction of melatonin and the internal standard from the aqueous standards and urine specimens. In this study, urine was adjusted to different pH values (4,6,7,8,12) and the analytes extracted using chloroform. At the neutral pH 7 the absolute extraction efficiency was found to be the greatest for both melatonin and 6-methoxymelatonin (mean 90%, n=6, refer Table 2 for relative recovery), and the chromatograms of urine extracts were cleanest. Although, at alkaline pHs the extraction efficiencies were equally good, the chromatograms were dirty and complex.

Our observations of good recovery from the urine at neutral and alkaline pHs is similar to earlier work on plasma samples.[6] Mills et al.[8] extracted melatonin from a saturated boric acid solution of urine and reported an absolute extraction efficiency of about 69%.

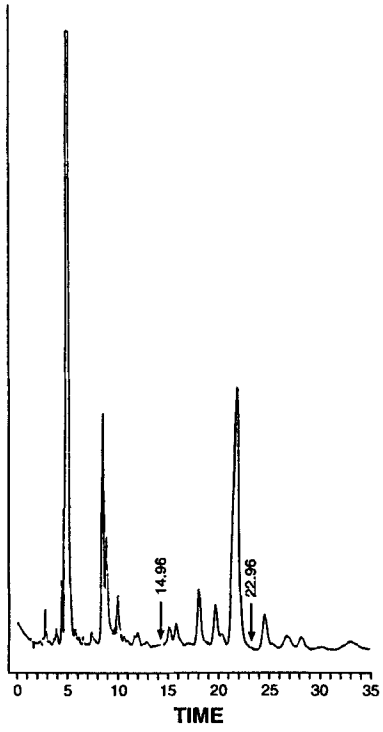

Figure 2. Chromatogram of an extract of urine stripped of melatonin and containing no 6-methoxymelatonin

RESULTS AND DISCUSSION

Representative chromatograms obtained in the work are shown in Figures 1-3. Figure 3 gives the chromatogram of a patient sample containing 57.6 pg/mL of melatonin, and Figure 2 is the chromatogram of stripped urine without the internal standard and shows the absence of interfering peaks at 14.9 min (retention time of 6-methoxymelatonin) and the blank spot at 22.96 min, the retention time of melatonin.

Table 1 gives a list of indole compounds that were checked for possible interferences in the work, and the study demonstrated the specificity of this method for melatonin. Mills et al.[8] in their work on urine samples, reported that 5-methoxy tryptophol and tryptophol eluted close to melatonin peak and our observations confirm the same. Table 2 gives details of the performance characteristics of the method. This precision and recovery

Table 1

Retention Times of Different Indole Compounds in the Assay

Compounds	Retention Times (min)
Serotonin	2.00
N-methylserotonin	2.10
5-Hydroxy tryptophan	2.10
L-Tryptophan	3.00
5-Methoxytryptophan	3.20
5-Hydroxy indole-3-aceticacid	3.76
5-Hydroxytryptophol	4.14
Indole-3-acetaldehyde	4.74
Tryptamine	4.89
5-Methoxytryptamine	5.00
N-acetylserotonin	5.19
N-methyltryptamine	5.64
6-Hydroxymelatonin	7.27
N-acetyl-L-tryptophan	7.77
6-Methoxymelatonin	14.91
5-Methoxy indole-3-acetic acid	15.42
Indole-3-acetic acid	15.47
5-Methoxytryptophol	17.70
Tryptophol	18.60
Melatonin	22.98

study was performed by initially stripping urine free of melatonin by repeated extractions (n=4) with chloroform, verifying its blank nature and then spiking it to known concentrations. Our mean (n=6) inter-assay coefficient of variation is 4.3%, and the mean intra-assay coefficient of variation is 5.8%. The mean (n=6) relative recovery (with respect to internal standard) of melatonin using the above spiked samples is 96%, and absolute recovery was 90% (n=6).

All sample measurements were made using a series of aqueous standards covering the range 25 to 250 pg/mL, and the method is linear in the concentration range 0 to 1000 pg. Standard curves were obtained using regression method, and the mean correlation coefficient for the line was 0.996. A detailed report of the sample results obtained by the method will be published elsewhere.[12] Table 3 gives the results for both the evening and morning samples of patients in a condensed form.

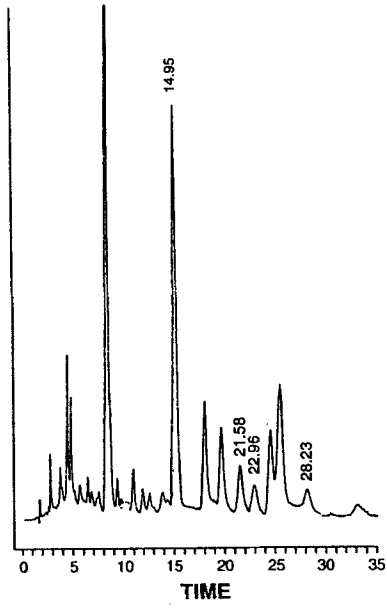

Figure 3. Chromatogram of a patient urine extract containing 57.6 pg/mL of melatonin and 50 ng of 6-methoxymelatonin

Table 4 gives a short summary and the detection limits of some of the recent HPLC electrochemical or fluorometric detector methods for the assay of melatonin. Two different claims[6,9] have been made using the same and efficient electrochemical detector (Environmental Science Associates, Bedford, MA). There is only limited scope to improve the detection limit using even an efficient electrochemical detector. The factors to achieve this include, using larger sample volumes for extraction, smaller size (internal diameter) columns, mobile phase composition, column heating, increase in mobile phase buffer concentration, and addition of ion-pairing reagents. But, with a fluorometric detector, the scope for improved detection limits are much greater because there are many other factors besides those listed above. In a dilute solution, the magnitude of fluorescence intensity depends upon the concentration of the fluorophor, the path length of the flow cell, the intensity of the exciting radiation, absortivity of the substance and its quantum efficiency.[13] Many workers, familiar with amino acids analyses, know that the sensitivity of fluorescent tag method reaches the femto moles range, whereas, with the electrochemical method it is in the range of pico moles. Another advantage with a fluorometric detector is that the baseline stabilizes very fast and it could

Table 2

Precision and Recovery Data for Melatonin Using Stripped Urine

Concn. Added	Conc. Measured Mean * ± S.D. (pg/mL)	C.V. %	Recovery Mean* %
	Inter-assay		
25	23 ± 1.15	5.0	92
62.5	60 ± 2.76	4.6	96
125	120 ± 4.32	3.6	96
250	237 ± 9.48	4.0	95
	Intra-assay		
25	22 ± 1.98	9.8	88
62.5	64 ± 3.07	4.8	102
125	128 ± 5.25	4.1	102
250	245 ± 11.0	4.5	98

* n = 6

Table 3

Urine Melatonin Concentrations in Our Patients

Mel. Concn. Range pg/mL	Frequency of Repetitions AM Results	PM Results
5 - 10	0	9
11 - 20	0	12
21 - 35	0	3
36 - 50	6	1
51 - 75	7	1
76 - 120	6	1
121 - 180	4	1
181 - 280	3	0

Table 4

Highlights of a Few Recent HPLC EC/FLU Methods for Melatonin Assay

Ref	Detector	DL	Samp	Recov %	Remarks
9	EC-ESA	1pg	2mL	90	Cell was set at (0.5 mV). Not the optimum potl.
6	EC-ESA	4pg	NK	97	Cell at optimum potl. 0.75 mV
7	EC-amp	15pg	2mL	78	Col 5 x 0.46, RT mel 25 min
8	FLU	30pg	8mL	69	Col 25 x 0.46, at 60°C, urine pH not optimized for extraction
5	FLU	6 pg	4mL	76	Gradient method; col at 30°C
*	FLU	0.5Pg	8mL	96	"Waters" Flu detector (Model 474, with a 16 μL flow cell) internal standard method

amp: amperometric detector; Col: column; DL: detection limit; NK: not known; potl.: potential; RT: retention time; *this method.

take several hours to stabilize an electrochemical detector. Thus the careful selection of a very good fluorometric detector for the melatonin assay is one of the main keys for a successful and sensitive HPLC method. We found that the fluorometric detector by "Waters" Model 474 with a 16 μL flow cell to be one of the best, and the on column detection limit for melatonin in our method is 0.5 pg (gave a peak height of 2 cm at an attenuation of 1).

The two recent fluorometric methods[5,8] for melatonin have other drawbacks besides the lack of sensitivity of their fluorometric detector. Both of them do not use an internal standard. Also, Penniston et al.'s method is a gradient method, and the run time per sample is 48 min, and the column is heated to 30°C, and the recovery of their solid phase extraction method is ~70%. Mills et al.[8] heated their column to 60°C.

In summary, when there is a choice between fluorometric and electrochemical detection for a compound, the former is far superior and several orders of magnitude more sensitive than the latter and easier to use. Our fluorometric HPLC assay for melatonin improves upon the earlier ones[5,8] by using a very sensitive detector and a good internal standard. The method has good accuracy (96%) and precision (C.V. ~5%).

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Received December 20, 1995
Accepted January 10, 1996
Manuscript 4052