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HPTLC Method for the Analysis of Melatonin in Bulk and Pharmaceutical Formulations

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A simple, precise and stability-indicating high performance thin-layer chromatographic method of analysis of melatonin both as bulk drug and in pharmaceutical formulation was developed and validated. The method employed TLC aluminium plates precoated with silica gel 60F-254 as the stationary phase and mobile phase comprising toluene:ethyl acetate:formic acid (5:4.5:0.5 v/v). Densitometric analysis of melatonin was carried out in the absorbance mode at 290 nm. The linear regression analysis data for the calibration plots showed good linear relationship with r = 0.9989, with respect to peak area, in the concentration range 100-600 ng/spot. The method was validated for precision, specificity, recovery and robustness. The recovery of the drug from tablets carried out by standard addition method was found to be 99.72 ± 0.682. Statistical analysis proves that the method is repeatable, selective and accurate for the estimation of the drug. Forced degradation studies showed the effect of variations in pH, UV light and high temperature on the stability of melatonin. As the proposed method could effectively separate the drug from its degradation products, it can be employed as a stability-indicating method.

Key Words: Melatonin, HPTLC, Stability indicating.

INTRODUCTION

Melatonin is the principle neurohormone of the vertebrate pineal gland and is produced mainly at night[1]. It is an indoleamine and a derivative of the amino acid, tryptophan. Chemically, it is N-[2-(5-methoxy-1*H*-indol-3-yl)ethyl]acetamide[2]. It plays a key role in body rhythms and jet lag; hence mediates physiological, endocrinological and behavioural processes[3].

A variety of methods have been proposed and assessed to detect and quantify melatonin in body fluids and pharmaceutical formulations. The different techniques in the literature survey include spectrophotofluorometric[4] and spectrophotometric[5,6] methods, radio immunoassay[3,7], GC-MS[8-10], HPLC[1,11-15], linear scan voltammetric method[1], amperometric method[16] and differential-pulse voltammetry[17]. Reported HPLC methods used different

detectors like electrochemical[1,13,14], ultraviolet[11,15] and fluorescence[11-13]. But chromatographic techniques demand a lot of time and expertise in their operation at higher cost. Moreover, none of them is stability-indicating method. Forced decomposition studies are carried out to generate degradation products of the drug. The International Conference on Harmonization (ICH) guidelines explicitly require conduct of forced decomposition studies under a variety of conditions like pH, light, oxidation, dry heat, *etc.* and separation of drug from degradation products[18]. Thus, there is a need to develop a simple, rapid, sensitive and cost-effective technique. In these respects, high performance thin layer chromatography is an alternative technique to HPLC and other analytical methods, which is also more accurate, precise, reproducible and economical.

The primary objective of the study is to develop a new HPTLC method for the analysis of melatonin in bulk drug, carry out stress studies and accurately quantitate the active ingredient without interference from degradation products. Further, to validate the developed method as per ICH guidelines. Finally, the developed method was applied to the analysis of commercially available pharmaceutical dosage form of melatonin.

EXPERIMENTAL

Melatonin bulk drug was obtained as a gift sample from Dabur Research Foundation, Sahibabad. All other chemicals and reagents were of AR/HPLC grade. The instrument used in the present study was CAMAG-HPTLC system comprising CAMAG LINOMAT IV automatic sample applicator, CAMAG TLC Scanner III with WINCATS software; CAMAG twin through chambers were used.

Experimental chromatographic conditions: Stationary phase: silica gel 60 F_{254} TLC precoated aluminium plates, 200 µm layer thickness; mobile phase:toluene:ethyl acetate:formic acid (5:4.5:0.5 v/v); chamber saturation time: 10 min; sample application: 6 mm band; separation technique: ascending; temperature: 20 ± 5 ºC; migration distance: 75 mm; scanning mode: absorbance; detection wavelength: 290 nm; source of radiation utilized: deuterium.

Calibration curve: A stock solution of melatonin (100 µg/mL) in methanol was prepared by dissolving 10 mg of melatonin in 100 mL of methanol. Different volumes of stock solution 1, 2, 3, 4, 5 and 6 µL were spotted in duplicate on TLC plate by microlitre syringe with the help of automatic sample applicator, to obtain concentrations of 100, 200, 300, 400, 500 and 600 ng/spot of melatonin, respectively. The plates were developed in the twin trough chamber saturated with the mobile phase, dried and densitometrically scanned at 290 nm. The data of peak height/area *vs.* drug concentration were treated by linear least-square regression.

Validation of the proposed method

Precision: Repeatability of sample application and measurement of peak area were carried out by the proposed method using six replicates of the same spot (400 ng/spot of melatonin). The intra- and inter-day variation for the determination of melatonin was carried out at three different concentration levels of 200, 600 and 3000 ng/spot.

Robustness: By altering various experimental conditions like mobile phase composition, amount of mobile phase, plate treatment, time from spotting to chromatography and time from chromatography to scanning, the effects on the results were examined. It was carried out at different concentration levels of 200, 600 and 3000 ng/spot, in triplicate.

Limit of detection (LOD) and limit of quantitation (LOQ): In order to estimate the LOD and LOQ, blank methanol was spotted six times on the TLC plate and then developed, sprayed and scanned in a similar way as that for calibration curve and then signal-noise ratio was determined.

The detection limit (DL) may be expressed as:

$$DL = \frac{3.3\ \sigma}{S}$$

where, σ = the standard deviation of the response and S = the slope of the calibration curve.

The quantitation limit (QL) may be expressed as:

$$QL = \frac{10\ \sigma}{S}$$

Recovery studies: The recovery study was performed to determine if there is positive or negative interference from excipients present in the formulation. The accuracy of the method was ascertained on the basis of the recovery study by applying the standard addition method to the preanalyzed sample. The analyzed samples were spiked with extra 80, 100 and 120 % of the standard melatonin and the mixtures were analyzed by the proposed method. The experiment was conducted in quadruplicate. This was done to check for the recovery of the drug at different levels in the formulation.

Analysis of melatonin in pharmaceutical formulation: 20 Tablets (MELOSET-labeled to contain melatonin 3 mg per tablet) were weighed and finely powdered. The powder equivalent to 3 mg of melatonin was accurately weighed and transferred to a 100 mL volumetric flask. The drug from the powder was extracted with 30 mL methanol. To ensure complete extraction of the drug, it was sonicated for 0.5 h and volume was made up to 100 mL with methanol. The resulting solution was filtered using membrane filter (pore size 0.2 mm). 5 µL of the filtered solution (150 ng/spot) was

applied on TLC plate followed by development and scanning at 290 nm. The analysis was carried in quadruplicate. The possibility of excipient interference in the analysis was studied.

Forced degradation of melatonin

Acid and base induced degradation: The stock solution of melatonin (100 µg/mL) was prepared by dissolving 10 mg of the drug in 100 mL of methanol taken in a 100 mL standard volumetric flask. From the prepared stock, 15 mL each was pipetted out separately into two tubes and 5 mL of 1 M HCl and 1 M NaOH was added, respectively. These mixtures were refluxed for 2 h at 70 °C in the dark in order to exclude the possible degradative effect of light. The resultant solutions were filtered and applied on the TLC plate in triplicate (4 µL each). The chromatograms were run as described earlier.

Hydrogen peroxide induced degradation: From the prepared stock solution, further pipetted out 15 mL and to it was added 5 mL of 30 % (v/v) H_2O_2. The mixture was kept in boiling water bath for 10 min to completely remove excess of H_2O_2 and then refluxed for 2 h at 75 °C. The resultant solution was filtered, 4 µL was applied on the TLC plate in triplicate and the chromatograms were run.

Photochemical degradation: The methanolic stock solution of the drug was exposed to UV radiation at 254 nm for 24 and 48 h. The resultant solution was filtered, applied on the TLC plate in triplicate (4 µL each) and then the chromatograms were run.

Dry heat degradation: Powdered melatonin drug was stored at 80 °C for 3 h under dry heat conditions. The degraded products were then resolved and dissolved in methanol. The solution was filtered, 4 µL was applied on the TLC plate in triplicate and the chromatograms were run.

In all the degradation studies, the average peak area of melatonin after application (400 µL/mL) of the three replicates was obtained.

RESULTS AND DISCUSSION

Various solvent systems were evaluated to arrive at an optimum resolution of pure drug and degraded products. Good resolution and reproducible R_f of 0.44 was obtained with the mobile phase consisting of toluene: ethyl acetate:formic acid (5:4.5:0.5 v/v). Moreover, this solvent system gave dense, compact and well-separated spots of the drugs from the degraded samples. Densitometric quantitation was carried out in the absorbance mode at 290 nm and symmetrical, well-resolved, well-defined peaks (Fig. 1) were obtained for melatonin. The linear regression data for the calibration plots (n = 3) in Table-1 showed good linear relationship over the concentration range 100-600 ng per spot with respect to peak area and coefficient of correlation value, r = 0.9989 (Fig. 2). The proposed HPTLC method was

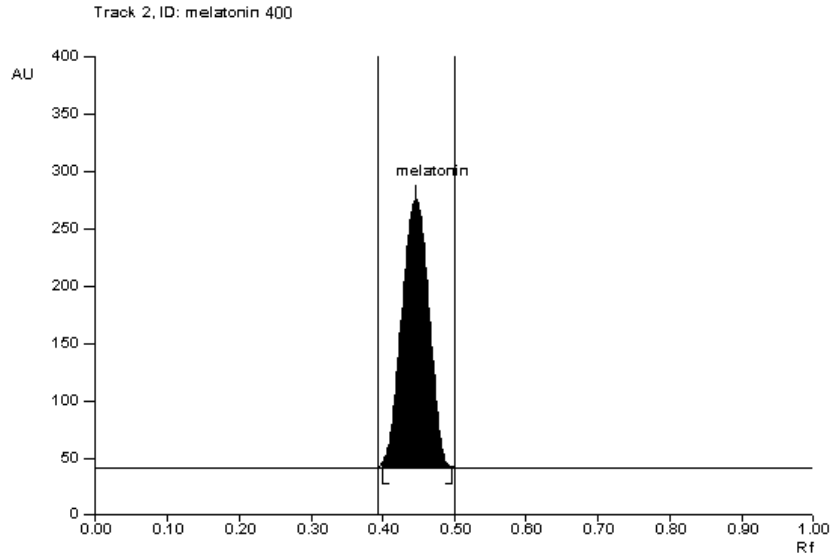

Fig. 1. Typical HPTLC chromatogram of melatonin

TABLE-1
DATA FOR CALIBRATION CURVE OF MELATONIN (n = 3)

Amount of standard (ng/spot)	Mean area ± SD
100	3043.787 ± 7.78
200	5333.200 ± 5.58
300	7316.240 ± 5.88
400	8888.873 ± 7.52
500	10127.390 ± 7.95
600	11482.700 ± 13.71

validated as per ICH guidelines. The precision of the method was evaluated by repeatability of sample application and measurement of peak area using six replicates of the same spot (400 ng/spot of the drug) with % RSD of 0.0885 for method precision studies and 0.118 for system precision studies. The intra-day and inter-day precision data are shown in Table-2. The robustness of the method was evaluated by altering the various experimental conditions. This was carried out at three different concentration levels of 200, 600 and 3000 ng/spot, in triplicate for melatonin with % RSD values of 0.41 (Table-3). The limit of detection and limit of quantitation for melatonin are 15 ng/spot and 45 ng/spot, respectively. The accuracy of the method was evaluated by percentage recovery (by standard addition) of the drug. The average recovery was found to be 99.72 ± 0.682 (Table-4). The results of the method lying in the prescribed limit of 98-102 % show that the

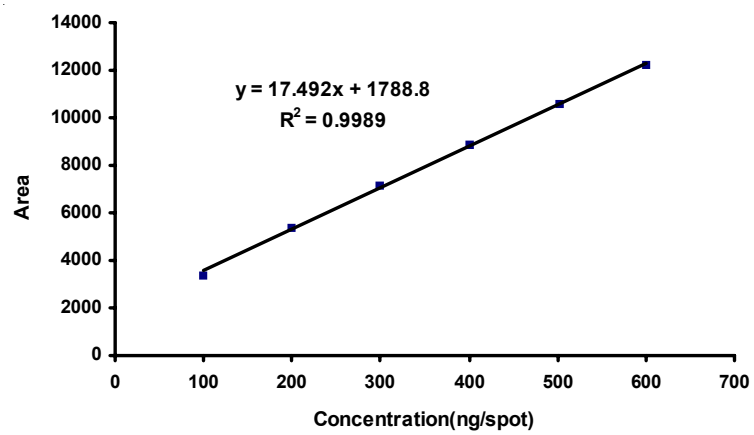

Fig. 2. Calibration plot for melatonin

TABLE-2
INTRA- AND INTER-DAY PRECISION DATA OF THE METHOD FOR MELATONIN (n = 6)

Amount (ng/spot)	Intra-day precision			Inter-day precision		
	Mean area	± SD	RSD (%)	Mean area	± SD	RSD (%)
200	6328.08	8.58	0.135	6348.80	11.60	0.182
600	10404.56	11.61	0.111	10495.01	10.83	0.103
3000	17015.00	15.68	0.092	17015.17	14.59	0.086

TABLE-3
ROBUSTNESS DATA OF THE METHOD FOR MELATONIN (n = 3)

Parameters	RSD (%)			Mean RSD (%)
	200	600	3000	
Mobile phase composition	0.45	0.37	0.58	0.46
When 15 mL of mobile phase was used	0.32	0.25	0.47	0.34
When plate was activated for 40 min	0.38	0.27	0.49	0.38
After 5 min of spotting, development of plate was carried out	0.48	0.32	0.50	0.43
After 10 min of development of plate, scanning was carried out	0.47	0.35	0.50	0.44

method is free from interference of excipients. The method was also applied to the analysis of melatonin in its pharmaceutical dosage form (Table-5) and the drug content was found to be 99.05 with % RSD of 0.367. It may be inferred that degradation of the drug had not occurred in the formulation that was analyzed by this method. Low values of % RSD indicate the suitability

TABLE-4
RECOVERY STUDIES DATA FOR MELATONIN (n = 3)

Excess drug added to analyte (%)	Theoretical content (ng)	Recovery (%)	RSD (%)
80	90	98.97	0.525
100	100	99.90	0.597
120	110	100.30	0.564

TABLE-5
ANALYSIS OF MARKETED FORMULATION OF MELATONIN BY HPTLC METHOD

Theoretical content (mg/tablet) label claim	Amount of drug recovered (mg)	Recovery (%)	Mean % recovery ± SD	RSD (%)
3	2.987	99.56	99.05 ± 0.364	0.367
3	2.974	99.13		
3	2.962	98.73		
3	2.959	98.63		
3	2.967	98.90		
3	2.981	99.36		

of the method for routine analysis of melatonin in its pharmaceutical dosage form. The forced degradation studies showed the effect of variations in pH, UV light and high temperature on the stability of melatonin (Table-6). The spots of the degraded product were well resolved from the drug spot.

TABLE-6
FORCED DEGRADATION OF MELATONIN

Sample exposure conditions	No. of degradation products (R_f value)	Melatonin remained (ng/spot) (±SD, n = 4)
Melatonin-1 M HCl	7 (0.01, 0.04, 0.36, 0.66, 0.69, 0.74, 0.87)	3.29 (± 3.45)
Melatonin-1 M NaOH	9 (0.01, 0.05, 0.09, 0.36, 0.66, 0.71, 0.86, 0.96, 0.97)	6.50 (± 8.74)
Melatonin-30 % H_2O_2	9 (0.03, 0.40, 0.64, 0.66, 0.71, 0.73, 0.80, 0.86, 0.97)	20.03 (± 4.82)
Melatonin-UV light	7 (0.03, 0.10, 0.18, 0.39, 0.64, 0.69, 0.87)	6.03 (± 7.53)
Melatonin-Dry heat	8 (0.04, 0.36, 0.64, 0.69, 0.72, 0.83, 0.86, 0.91)	4.28 (± 3.61)

The results obtained on analysis by the proposed method for melatonin revealed that the HPTLC method is simple, accurate, precise and reproducible for the estimation of the drug as pure drug powder and in pharmaceutical dosage form. Moreover, this method can be used for the routine analysis of melatonin in its commercially available marketed formulations.

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