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HIGH-PERFORMANCE THIN LAYER CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS QUANTITATION OF MELATONIN AND ZOLPIDEM IN TABLET FORMULATION

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ABSTRACT

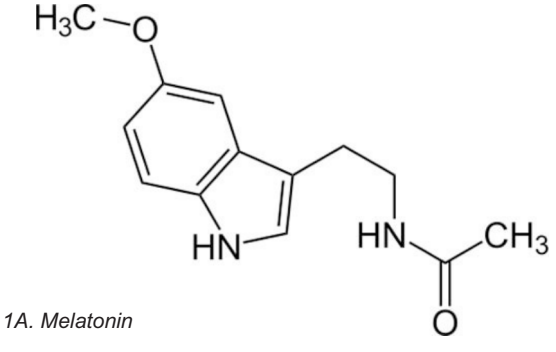
A simple, accurate and precise densitometry method for the simultaneous estimation melatonin (MEL) and zolpidem (ZOL) in pharmaceutical dosage forms has been developed and validated. Separation of drugs was carried out using toluene: n-butanol: glacial acetic acid: water (1:4:2:2v/v/v/v) as mobile phase on precoated Silica Gel 60F_{254} plates. The densitometry evaluation of spot was carried out at 254 nm. The R_f value for MEL and ZOL were found to be 0.87±0.01 and 0.59±0.01 respectively. The method was validated with respect to linearity, accuracy, precision and robustness as per the International Conference on Harmonization (ICH) guidelines. The drug response with respect to peak area was linear over the concentration range 150-900 ng/spot and 250-1500 ng/spot for MEL and ZOL respectively, limit of detection and limit of quantitation were found to be 14.96 ng/spot and 45.60 ng/spot for MEL and 19.53 ng/spot and 59.24 ng/spot for ZOL. The percentage recovery of MEL and ZOL were found be 99.04-99.35% and 98.11-98.77% respectively. The % RSD values for (intra-day RSD 0.26-0.56% and inter-day RSD 0. 36-0.78 %for MEL and intraday RSD 0.18-0.31% and inter day 0.23-0.31% for ZOL). It was observed that the proposed HPTLC method could be used for efficient analysis and monitoring of the MEL and ZOL in combined tablet dosage forms.

Key words: *Melatonin; Zolpidem; HPTLC; Validation.*

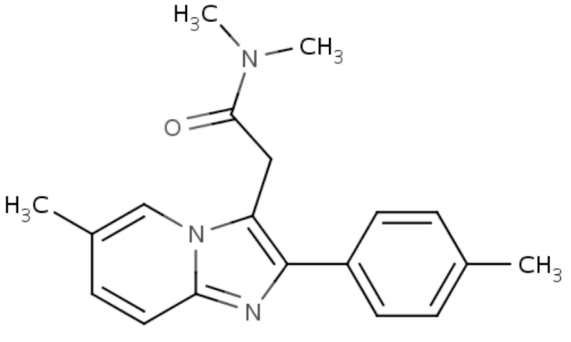
INTRODUCTION

Melatonin (MEL) is chemically, N-[2-(5-Methoxy-1*H*-indol-3-yl) ethyl] acetamide[1] (Fig.1A), clinically used in the treatment of cancer, immune disorder, cardio-vascular diseases, depression and sexual dysfunction. Zolpidem (ZOL) is chemically is an N,N,6-Trimethyl-2-(4-methylphenyl)imidazo[1,2-α] pyridine-3- acetamide[2] (Fig.1B). It is used for short-term treatment of insomnia, as well as some brain disorders. It is short-acting non benzodiazepine hypnotic of the imidazo pyridine class that potentiates gamma-amino butyric acid (GABA), an inhibitory neurotransmitter.

Literature survey reported that many analytical methods for quantitation of MEL by RP-HPLC[3-4] and by radio-immunoassay[5] in individual and in combination with other drugs. Several methods have been described for the determination of ZOL by UV spectrophotometry[6-7,] stability indicating HPLC [8-9,] bulk drugs and human plasma [10-11] reported for the simultaneous estimation of MEL and ZOL drugs[12]. However, there is no analytical method was reported for the simultaneous estimation of MEL and ZOL in combined dosage form. Our study, attempts to develop an accurate, precise, specific, linear, simple, rapid, and cost effective analytical method for the simultaneous estimation MEL and ZOL in combined tablet dosage forms by HPTLC method and to validate the method as per International Conference on Harmonization (ICH) guidelines[13-14.]



1A. Melatonin



1B. Zolpidem

Fig.1: *Chemical structure of (A) Melatonin and (B) Zolpidem*

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MATERIALS AND METHODS

Instrumentation

The samples were spotted in the form of bands of 6 mm using a Camag 100 µl microliter syringe on silica gel precoated aluminium plate 60 GF_{254}, (20 cm X 10 cm X 250 µm; E. Merck) The plates were prewashed with methanol and activated at 110º C for 5min (prior to chromatography). Space between two bands was maintained as 6 mm. A constant application rate of 100nl/s was employed Camag Linomet 5 Automatic TLC sample applicator. The slit dimension was kept at 5 mm X 0.45 mm and 10mm/s scanning speed was employed. The monochromator bandwidth was set at 20 nm, each track was scanned trice and the baseline correction was used. Linear ascending development was carried out in a 20 cm X 10 cm twin through glass chamber saturated with the mobile phase. The optimized chamber saturation time for mobile phase was 30 min at room temperature (25ºC±2) at relative humidity of 60±5%. The length of chromatogram run was 8 cm. Subsequent to the development, HPTLC plates were dried in current of air with the help of an air dryer in a wooden chamber with adequate ventilation. Densitometry scanning was performed on a Camag HPTLC III scanner in the reflectance absorbance mode at 254 nm and operated by WIN CATS software (V 3.1.0, Camag). The source of radiation utilized was deuterium lamp emitting continuous UV spectrum between 190 and 400 nm.

Reagents and chemicals

Melatonin and Zolpidem standard substance (sample of MEL, % purity 99.43% and ZOL, % purity 99.62%) were provided as a gift sample by Reltfen Pharmaceutical, Pondicherry. These samples were used without further purification. The tablet formulation (Brand name: Zolcalm-5; label claim containing MEL 3mg and 5mg) was used for the analysis. All chemicals and reagents of analytical grade were purchased from Merck chemicals Ltd., Mumbai, India.

Preparation of standard solution

MEL and ZOL stock solution was prepared in methanol and sonicated for 10 min to obtain standard stock solution concentrations of 1000µg/ml. From this 1.5ml of MEL and 2.5 ml of ZOL were transferred to 10 ml flask and diluted to volume with methanol to obtain the final concentration contains 150 µg/ml for MEL and 250µg/ml for ZOL.

Preparation of sample solution

To determine the content of MEL and ZOL simultaneously in a conventional tablet (Zolcalm-5, Label claim 3 mg MEL and 5 mg ZOL per tablet), twenty tablets were accurately weighed, and average weight determined and ground to fine powder. A quantity of powder equivalent to 3 mg of MEL, 5 mg of ZOL was transferred into a 10 ml volumetric flask containing 6 ml of methanol, sonicated for 30 min and diluted to mark with methanol. The resulting solution was filtered using Whatman paper No.41. So, the solution was further dilute with found to contain150 µg/ml and 250µg/ml for MEL and ZOL.

Analysis of tablet formulation

The resultant solution was found to be contain 150 µg/ml and 250 µg/ml for MEL and ZOL and two µl of this solution was applied on TLC plate and developed in mobile phase of toluene: n-butanol: glacial acetic acid: water (1:4:2:2 v/v/v/v) . The analysis was repeated for three times.

Development and Validation of HPTLC method

Validation of the optimized HPTLC method was carried out with respect to the following parameters according to ICH norms.

Linearity and range

From the standard stock solution (150 µg/ml of MEL and 250µg/ml of ZOL), 1-6 µl were spotted on the TLC plate to obtain the concentration 150-900 ng/spot for MEL and 250-1500 ng/spot for ZOL. The plate was then developed using the previously described mobile phase and the peak areas were plotted against the corresponding concentration to obtain the calibration curve.

Precision

The precision of the method was determined in terms of intra-day and inter-day variation (%RSD) was assessed by analyzing standard drug solutions within the concentration of calibration range, three times on the same day. Inter-day precision (%RSD) was assumed by analyzing standard solutions within the calibration range on three consecutive days.

Accuracy (%Recovery)

Accuracy of the method was carried out by applying the proposed method to the test sample (MEL and ZOL combination tablet). To which a known amount of MEL and ZOL sample corresponding to 80%, 100% and 120% of label claim (Standard addition method) and analyzed by running chromatogram in an optimized mobile phase. This was done to check for the recovery of the drug at different levels in the formulation.

Limit of detection and limit of quantification

The detection limit of assay is the lowest concentration that can be detected. The quantitation limit is the lowest concentration that can be quantified with acceptable precision. The quantitation limit is the lowest level of analyte that can be reported. The ICH guidelines suggest three different methods for determining the detection and quantitation limits. The visual determinations, signal-to-noise ratio determination and standard deviation and slope method. The signal- to- noise ratio method low levels of the analyte to a blank or background sample, measured signals from samples with known low concentrations of analyte with those of blank samples and subsequently establishing the minimum concentration at which analyte can be reliably detected. LOD is calculated by, LOD = 3 * SD/slope of calibration curve. LOQ can be calculated as follows, LOQ=10 * SD/slope of calibration curve.

Robustness of the method

Robustness of the proposed TLC densitometry method was determined to evaluate the interference of small deliberate changes in the chromatographic conditions

during determination of MEL and ZOL. Robustness was determined by changing in mobile phase, development distance and saturation time.

Specificity

The specificity of the method was determined by analyzing standard drug and test samples. The identities of the spots for MEL and ZOL were confirmed by comparing the R_f and spectra of the spots with that of the standard. Peak purity of MEL and ZOL was assessed by comparing the spectra at three different point leads the peak start(S), peak apex (M), and peak end (E) positions of the spot. The wavelength 254 nm was selected for densitometry scanning.

RESULT AND DISCUSSION

Thin layer chromatographic separation procedure was optimized using different solvent system and chromatographic conditions for simultaneous estimation of MEL and ZOL. Initially, on the plates, two µl of standard solution was applied as a band of 8 mm width. Initially, plates were developed by using solvents like toluene, hexane, methanol, chloroform, ethyl acetate, acetone and acetonitrile. Based on the initial observation, solvent systems like methanol: ethyl acetate: water, methanol: chloroform: acetone were tried. On the basis of this observation, the toluene: acetonitrile: ethyl acetate: methanol: ammonia system was tried in various ratios. After several trials, the mixture of toluene: n-butanol: glacial acetic acid: water (1:4:2:2 v/v/v/v) as mobile phase was chosen as the mobile phase for analysis and no immiscibility issues were found with the selected mobile phase combination. Other chromatographic conditions like chamber saturation time, run length, sample application rate and volume, sample application positions, distance between tracks and detection wavelength were optimized to give reproducible R_f values, better resolution and symmetrical peak shape for the two drugs scanning was performed at 254 nm for the detection of MEL and ZOL with R_f values of 0.87 and 0.59, respectively. (Fig. 2)

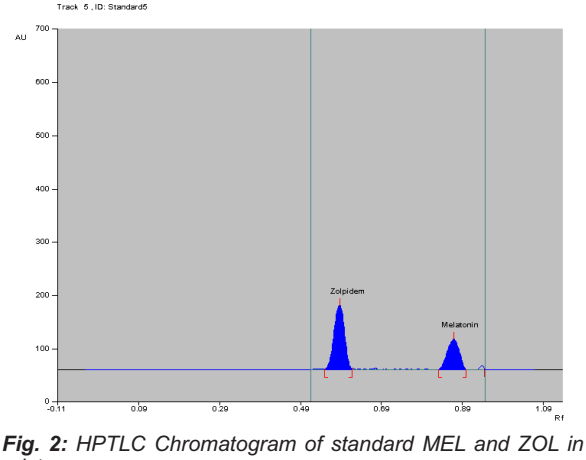

Fig. 2: *HPTLC Chromatogram of standard MEL and ZOL in mixture*

Analysis of tablet formulation

Two µL of sample solution was applied to an HPTLC plate to give final amount of 300 ng/spot and 500ng/spot MEL and ZOL. After the chromatographic development, peak areas of the bands were measured and amount of each drug was estimated from the respective calibration plots. The procedure was repeated three times. The average assays (n=3) were 98.86 ± 0.13 and 98.29 ± 0.54 for MEL and ZOL respectively. Experimental results for the amount of MEL and ZOL, the percentage of label claims were in good agreement with the label claims and thereby suggesting that there is no interference from any of the excipients which are normally present in tablets and the results are tabulated in Table 1.

Table 1: *Analysis of the tablet Formulation*

S.No	Drug	Label claim(mg/tablet)	Amount found(mg)	Label claim*(%)mean[a]±S.D[b]	% RSD
1	Melatonin	3mg	2.96	98.86±0.13	0.14
2	Zolpidem	5mg	4.91	98.29±0.54	0.55

[a]*=Average mean of three determinations,* [b] *=Standard derivation*

Linearity

Linear regression data for the calibration plots revealed good linear relationships between area and concentration over the ranges 150-900 ng /spot for MEL and 250-1500 ng /spot for ZOL. The linear regression equations were Y=2.0756X+113.67 for MEL and Y=2.0624X+358.01 for ZOL respectively. The calibration plots were with regression (r^2) being 0.9809 and 0.9929 for MEL and ZOL respectively in Table 2 (Fig 3 and 4).

Table 2: *Summary of Validation parameter*

Validation parameter	Melatonin	Zolpidem
Detection wavelength(nm)	254	254
Linearity range (ng /spot)	150-900	250-1500
Slope	2.0756X	2.0624X
Intercept	113.67	358.01
Correlation coefficient(r2)	0.9809	0.9929
LOD	14.96	19.53
LOQ	45.69	59.24
Specificity	Specific	Specific

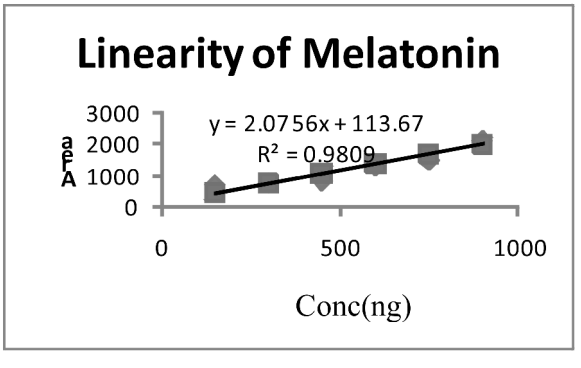

Fig.3: *Linearity curve of MEL at 254 nm*

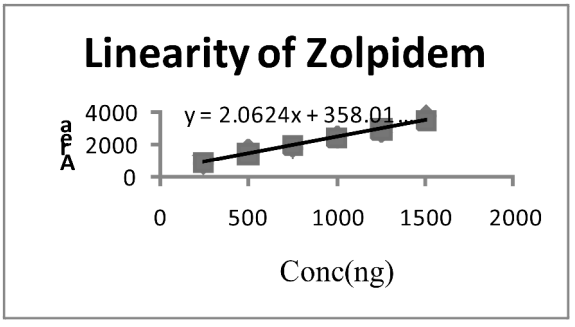

Fig.4: *Linearity curve of ZOL at 254 nm*

Precision

Results from determination of intraday and interday precision, were expressed as SD and relative standard deviation (RSD %). The precision of the proposed method were < 2%, confirming that the method was sufficiently precise in Table 3 & 4.

Table 3: *Intra-day and inter-day study of melatonin*

Concentration (ng/spot)	Intra-Day area Mean(n=3)±SD	%RSD	Inter-Day Area Mean(n=3)±SD	%RSD
300	730.04±1.90	0.26	721.53±2.66	0.36
450	759.73±4.11	0.54	782.56±6.15	0.78
600	1317.6±4.77	0.36	1331.43±6.08	0.45

Table 4: *Intra-day and inter-day study of zolpidem*

Concentration (ng/spot)	*Intra-Day area Mean(n=3)±SD*	*%RSD*	*Inter-Day Area Mean(n=3)±SD*	*%RSD*
500	1643.36±5.23	0.31	1613.86±3.76	0.23
750	1763.43±5.42	0.30	1788.30±5.67	0.31
1000	2402.23±4.34	0.18	2435.56±6.80	0.28

Recovery

The accuracy of the method was obtained on the basis of recovery studies performed by standard addition method with 80,100 and120% of the label claim each in triplicate. A known amount of each standard powder was mixed with sample of tablet powder and these were then analyzed as described above. The results from recovery analysis was found to be 100.13-100.25% with RSD 0.21-0.31% for MEL and 99.61-99.89% with RSD 0.24-0.38% for ZOL. Table 5.

Table 5: *Results from recovery studies*

Drug	Label claim	Amount Added (%)	Amount recovered(mg)	Recovery (%)	% RSD
Melatonin	3 mg	80	5.34	99.04±0.24	0.24
		100	5.95	99.26±0.38	0.38
		120	6.55	99.35±0.22	0.23
Zolpidem	5 mg	80	8.80	98.42±0.81	0.82
		100	9.80	98.11±0.25	0.26
		120	10.80	98.77±0.35	0.36

Robustness of the methods

The relative standard deviation of peak areas was less than 2%. These percent RSD indicates the robustness of the method in Table 6.

Specificity

The mobile phase resolved both the drugs very efficiently, as shown in Fig 2. The peak purity of MEL and ZOL were assessed by comparing their respective spectra at the peak start, apex, and peak end positions of the spot, r^2= 0.9973 and r^2= 0.9995. A good correlation was also obtained between the standard and sample spectra of MEL and ZOL respectively. Also excipients from formulation were not interfering with the assay. Also excipients from formulation were not interfering with the assay.

Table 6: *Robustness of the method*

Experimental conditions	Melatonin		Zolpidem	
	SD	%RSD	SD	%RSD
Mobile phase composition				
Toluene: n-butanol: acetic acid: Water(1:4.2:2:2)	8.10	1.05	8.90	0.49
Toluene: n-butanol: acetic acid: Water(1:3.8:2:2)	9.18	1.39	8.10	50
Development distance				
7 cm	7.17	1.16	8.05	0.47
7.5 cm	6.38	0.87	7.12	0.39
8 cm	6.07	0.76	6.79	0.37
Duration of saturation				
20 min	8.02	1.07	8.16	0.47
25 min	6.11	0.73	7.44	0.41
30 min	7.95	0.89	4.56	0.24

CONCLUSION

The developed HPTLC technique is simple, accurate, precise and reproducible for simultaneous determination of melatonin and zolpidem in pharmaceutical dosage forms. In this method reduces cost per analysis and analysis time, suitable for routine analysis of pharmaceutical formulations in quality-control laboratories. The major advantage of HPTLC is that several samples can be run simultaneously using a minimum sample preparation and small quantity of mobile phase.

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