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HPTLC-DENSITOMETRIC ANALYSIS OF ALPRAZOLAM AND MELATONIN IN THEIR COMBINED TABLET DOSAGE FORM

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ABSTRACT
The present study describes development and subsequent validation of stability indicating HPTLC methods for simultaneous estimation of Alprazolam (ALP) and Melatonin (MELA) in their combined formulation. The proposed HPTLC, separations were performed on silica gel 60 F_{254} using Ethyl acetate: chloroform: hexane: glacial acetic acid (6 : 3 : 4 : 0.7 v/v) as mobile phase. The compact bands of ALP and MELA at Rf 0.06 and 0.19 respectively were scanned at 266 nm. Linear regression analysis revealed linearity in the range of 50 to 300 ng/band for ALP and 300 to 1800 ng/band for MELA respectively. For both the methods, dosage form was exposed to thermal, photolytic, acid, alkali and oxidative stress. The methods distinctly separated the drugs and degradation products even in actual samples. In conclusion, the proposed HPTLC methods were appropriate for routine quantification of ALP and MELA in tablet formulation.

KEYWORDS: The present study MELA in tablet formulation.

INTRODUCTION
Alprazolam and Melatonin which has been found effective in the treatment of Anti-anxiety, also useful in treating psychiatric disorder.

Alprazolam: The chemical name of Alprazolam is 8-chloro-1-methyl-6-phenyl-4H-S-triazolo [4,3-α][1,4]benzodiazepine. It has a molecular formula of $C_{17}H_{13}ClN_4$ and molecular weight of 308.76 g/mol. It is a white to off-white crystalline powder and soluble in methanol or ethanol, sparingly soluble in acetone and freely soluble in chloroform. Alprazolam and other benzodiazepines act by enhancing the effects of gamma-amino butyric acid (GABA) in the brain. It possesses anti-anxiety or other psychiatric disorder effect. It is official in IP, Merck Index, Clarkes analysis of Drugs and Poisons.[1-3]

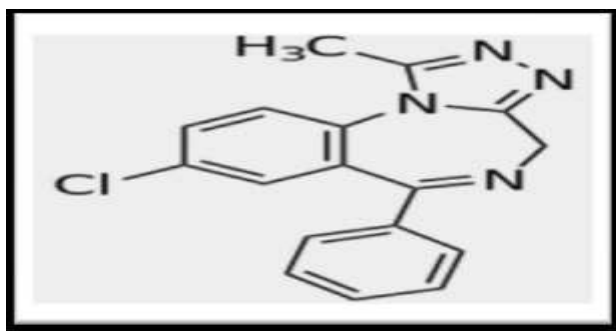


Figure. 1: Structure of Alprazolam.

Melatonin
The chemical name of Melatonin is N-[2,(5-Methoxy-1H-indol-3yl)ethyl]acetamide. It has a molecular formula of $C_{13}H_{16}N_2O_2$ and molecular weight of 232.278 g/mol. It is pale yellow powder and soluble in water, ethanol, methanol, chloroform and very slightly soluble in petroleum ether. Melatonin is a hormone synthesized in the pineal gland and to a lesser extent in the retina. It induced-immuno-opioids (MIIO) mediate the immune enhancing and anti-stress effects of melatonin. It is official in Merk Index.[4-6]

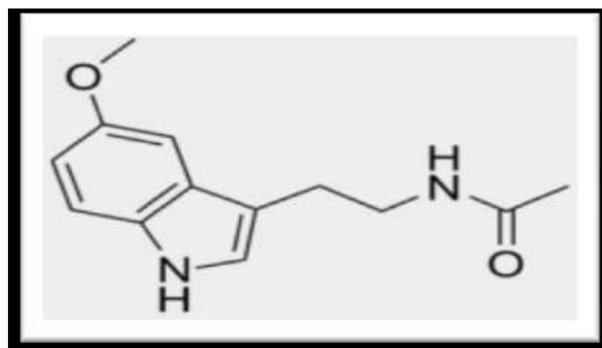


Figure. 2: Structure of Melatonin.

Literature review reveals various methods are reported for determination of Alprazolam and Melatonin in alone are in combination with other drugs, by UV-spectrophotometric[7,8,9], RP-HPLC[10-12] and HPTLC[14-

[15] methods are reported in literature. . But, no method is available for simultaneous determination of Alprazolam and Melatonin. The objective of this study was to develop a fast, precise, accurate, rugged and robust HPTLC method for simultaneous determination of Alprazolam and Melatonin in tablets. Criteria employed for assessing suitability of proposed method was cost effectiveness and speed of analysis.

MATERIALS AND METHODS

Chemicals: Alprazolam and Melatonin as pure standard reference drugs were obtained from Micro Labs. Ltd., Himachal Pradesh, India. Analytical grade ethylacetate, chloroform, hexane and glacial acetic acid(97 to 98% v/v) were obtained from M/S.Merk, Mumbai, India.

Formulation: Strensil – 0.5 TAB contains Alprazolam 0.5 mg, Melatonin 3 mg combination tablets were purchased from local market.

Intrumentation

In HPTLC, chromatographic separation of drug was performed with silica gel 60 F 254 (10.0 × 10.0 cm with 250mm layer thickness) from E. Merck, Germany. Samples were applied as 8mm bands by means of Camag 100 μL, sample syringe (Hamilton, Switzerland) with Linomat 5 applicator (Camag, Switzerland). Densitometric scanning was performed in the absorbance/ reflectance mode at 279 nm using Camag TLC scanner 3 with deuterium source, slit dimension settings of length 2 mm, width 0.1 mm, monochromator band width 30 mm, and scan rate of 4mms−1. Win CATS software (V 1.4.2, Camag, Switzerland)was used for scanner control and data processing.

Alprazolam (20µg/ml) and melatonin (120 µg/ml) Standard stock Solution

HPTLC, standard stock solution was prepared separately by dissolving accurately weighed 10mg each of ALP and MELA in 100mL methanol. From this stock solutions, suitable dilution was made using methanol to obtain a combination solution containing ALP(10 to 20µg/mL) and MELA (10 to 120µ g/mL) taking into consideration their ratio (1 : 6) present in combined tablet formulation.

Preparation of Sample Solution

Twenty tablets from each brand of one batch were accurately weighed; their mean weight was determined and powdered using glass mortar. For HPTLC, an amount equivalent to label claim of each active ingredient was accurately weighed and transferred into a suitable volumetric flask. The volume was adjusted with methanol, and the resultant solution was sonicated for 15min and filtered through 0.45 μm nylon filter. From the resulting solution, suitable aliquots were transferred into 100mL volumetric flask and completed to volume with methanol to have a final concentration of 120 μg/mL of MELA and 20 μg/mL of ALP, respectively. Calibration Curve for ALP and MELA. Semiautomatic spotter was used containing a syringe having capacity of 100 μL. Mixed stock solution having concentration of 20 μg/mL of ALP and 120 μg/mL MELA was filled in the syringe and under nitrogen stream, it was applied in the form of band of desired concentration range for each drug on a single plate having concentration of 50 to 300 ng/spot for ALP and 300 to 1800 ng/spot for MELA. Plate was developed using the above-mentioned conditions. Plots of peak area versus concentration for both drugs were obtained.

Table. 1: System suitability test parameter.

System suitability parameter	ALP	MELA
Peak purity	0.996	0.997
Rf value	0.06±0.0121	0.19±0.0104

Analysis of Marketed Formulation

Twenty tablets were weighed accurately; average weight was found and finely powered. A quantity equivalent to 20mg ALP and 120mg MELA was accurately weighed and transferred to volumetric flask of 100mL capacity. 80mL of methanol was transferred to this volumetric flask and sonicated for 20min to dissolve the drug. Resulting solution was filtered through What man filter paper (0.45 μ) into a 100mL volumetric flask. The flask was shaken, and volume was made up to the mark with methanol to give a solution containing 200 μg/mL of ALP and 1200 μg/mL of MELA. One mL of this aliquot was added to 10mL volumetric flask, and volume was made up to the mark with methanol to give a solution containing 20 μg/mL of ALP and 120 μg/mL of MELA. Now this prepared sample solution was applied on TLC plate, developed, dried in air, and photo metrically analyzed as described above. From the peak area obtained in the chromatogram, the amounts of both of the drugs were calculated.

Validation of the Method

Linearity and Range of the HPTLC Method

Calibration graphs were constructed by plotting peak areas versus concentrations of ALP and MELA, and the regression equations were calculated. The calibration graphs were plotted over 6 different concentrations in the range of 50–300 ng/spot for ALP and 300–1800 ng/spot for MELA by applying different volumes stock solution containing ALP and MELA (20 μg/mL of ALP and 120 μg/mL MELA). The calibration graphs were developed by plotting peak area versus concentrations (n = 6) with the help of the win CATS software.

Accuracy (Recovery)

Known amounts of standard solution of ALP (100, 150, and 200 ng/spot) and MELA (1200,1500 and 1800 ng/spot) for the HPTLC method were added to prequantitated sample solutions of tablet dosage forms. The amounts of ALP and MELA were estimated by applying values of peak area to the regression equations of the calibration graph.

Precision: Precisions of the proposed HPTLC methods were determined by analyzing mixed standard solution of ALP and MELA at 3 different concentrations (100, 150, and200 ng/spot for ALP and 600, 1500, and 1800 ng/spot for MELA) 3 times in the same day and in 3 different days. The results are reported in terms of coefficient of variance (CV).

Repeatability: Repeatability of method was assessed by applying the same sample solution 6 times on a plate with the automatic spotter using the same syringe and by taking 6 scans of the sample spot for both ALP and MELA(150 ng/spot of ALP and 1600 ng/spot of MELA) without changing the positions of the plate.

Specificity: The specificity of the method was ascertained by analyzing standard drug and sample. The band of ALP and MELA in sample was confirmed by comparing theRf and spectra of the band with that of standard. The peak purity of both drugs was assessed by comparing the spectra at 3 different levels, that is, peak start (S), peak apex (M), and peak end (E) position of the band.

Limit of Detection and Limit of Quantification

The limit of detection (LOD) and the limit of quantification (LOQ) of the drug were derived by using the following equations as per International Conference on Harmonization (ICH) guidelines which is based on the calibration curve.

Robustness: Sample solution was prepared and then analyzed with change in the typical analytical conditions like amount of mobile phase, proportion of mobile phase, saturation time, plate pretreatment and stability of analytical solution.

RESULTS AND DISCUSSION

Method Optimization: Several mobile phases were tried to accomplish good separation of ALP and MELA. Using the mobile phase Ethyl acetate : Chloroform: Hexane: Glacial acetic acid (6 : 3 : 4 : 0.7) (v/v/v/v) and 10 × 10 cm HPTLC silica gel 60 F254 aluminum-backed plates, good separation was attained with retention factor (Rf) values of 0.06 for ALP and 0.19 for MELA. A wavelength of 266nm was used for the quantification of the drugs. Figure 2 shows the detection of both of the drugs in their combined dosage format 266 nm by HPTLC method. Resolution of the peaks with clear baseline separation was found. Figure 3 shows the densitogram of mixture which has a clear baseline. Figure 4 showed a good linearity when overlapped and scanned between 200 nm to 400 nm. Figure 5 shows a 3D overlapped spectrum of both drugs which has good linearity. The system suitability test parameters for the developed method are shown in Table 1.

Validation of the Proposed Methods

Linearity

Figure 6 shows that linear correlation was obtained between peak areas and concentrations of ALP and MELA in the range of 50–300 ng/spot for ALP with R^2=0.996 and 300–1800ng/spot for MELA with R^2= 0.997,respectively, and data are shown in (Table 2).

Accuracy

The recovery experiments were performed by the standard addition method. The HPTLC method was found to be accurate with % recovery of 98.61–100.94% for ALP and 99.18–100.57% for MELA, respectively, (Table 3). The high values indicate that the method is accurate.

Repeatability

The CV values for ALP and MELA were found to be 0.69 and 0.35, respectively. The CV values were found to be <1%, which indicates that the proposed methods are repeatable. The CV values were found to be <2%, which indicates that the proposed method is precise.

Limit of Detection (LOD) and Limit of Quantification(LOQ)

LOD values for ALP and MELA were found to be 8.05 ng/spot and 87.64 ng/spot, respectively. LOQ values for ALP and MELA were found to be 24.41 ng/spot and 265.57 ng/spot, respectively. These data show that nanogram quantity of both drugs can be accurately determined (Table 4).

Specificity

Excipients (Starch) used in the specificity studies did not interfere with the estimation of either of the drugs by the proposed methods. Hence, the methods were found to be specific for estimation of ALP and MELA.

Robustness

Peak area and retention time variation were found to be <1%. Also, no significant change in peak area was observed during 24 hr. No decomposition was observed in either the first or second direction of the 2-dimensional analysis for both drugs on the HPTLC plate. Hence, the method was found to be robust for estimation of ALP and MELA.

Assay of the Tablet Dosage Form (ALP 50 mg and MELA 300mg per Tablet).

The proposed validated method was successfully applied to determine ALP and MELA in their tablet dosage form (Strensil). The results obtained for ALP and MELA was comparable with the corresponding labeled amounts (Table 5).

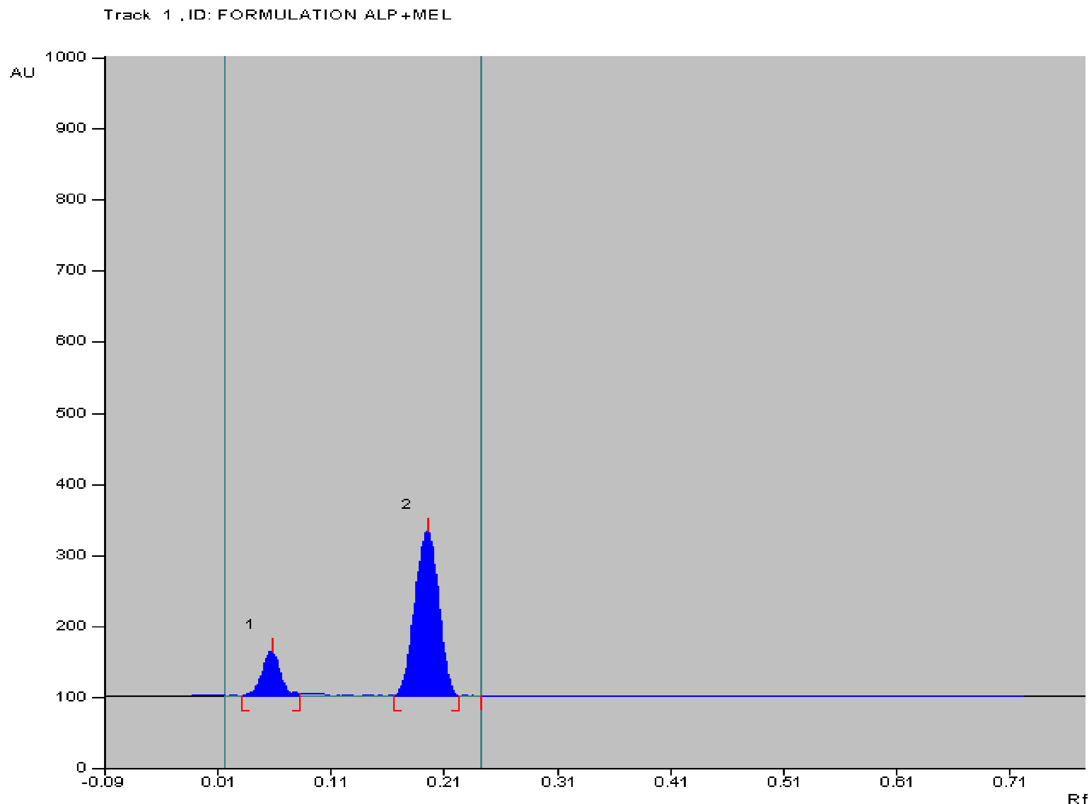


Fig 4 Densitogram of marketed formulation containing ALP and PCM 150ng/spot and 900 ng/spot, respectively.

Table. 2

Concentration(ng/spot)	Area mean ±S.D.(n=6)	CV
Result of calibration reading for ALP by HPTLC method		
50	286.2±2.34497	0.82
100	592.9±3.03905	0.5
150	862.6±11.41307	1.29
200	1140.8±10.546	0.92
250	1439.5±9.7660	0.67
300	1819.7±3.3964	0.18

Result of calibration reading for MELA by HPTLC method

300	1890.5±7.36668	0.4
600	3751.6±10.69726	0.28
900	5342.3±9.29899	0.17
1200	7582.9±96.56843	1.29
1500	9665.8±59.33787	0.61
1800	11498.4±114.3338	0.98

Table: 3 Determination of accuracy.

Amount added(ng/spot)			Amount recoverd(ng/spot)		%Recoverd±S.D	
% Level	ALP	MELA	ALP	MELA	ALP	MELA
80	160	960	161.9032	955.4662	101.14±0.67	97.35±0.54
100	200	1200	200.0667	1188.837	100.67±0.56	99.65±0.42
120	240	1440	237.8833	1425.222	98.78±0.56	98.68±0.32

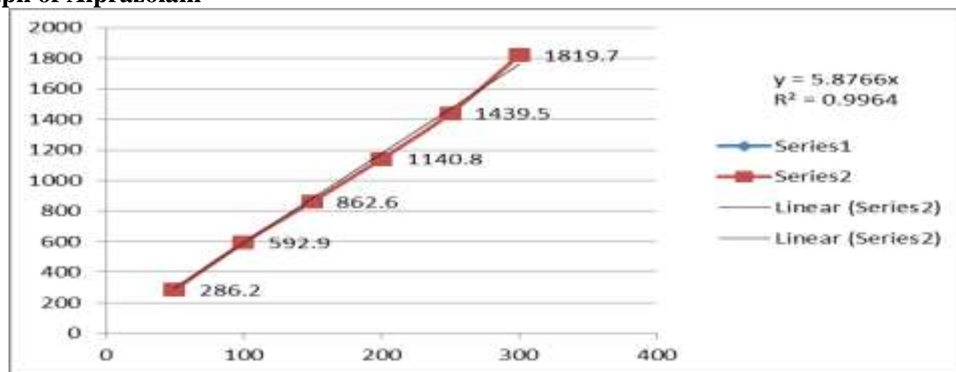
Table. 4: Summary of validation parameters of HPTLC.

Parameters	ALP	MELA
Recovery %	100.097-101.2	99.338-100.5
Repeatability (CV, n=6)	0.07	0.259
Precision (CV) Intraday(n=3) Interday(n=3)	 0.07 0.1056	 0.259 0.0583
Robustness	Robust	Robust
Solvent suitability	Suitable for 24hr	Suitable for 24hr

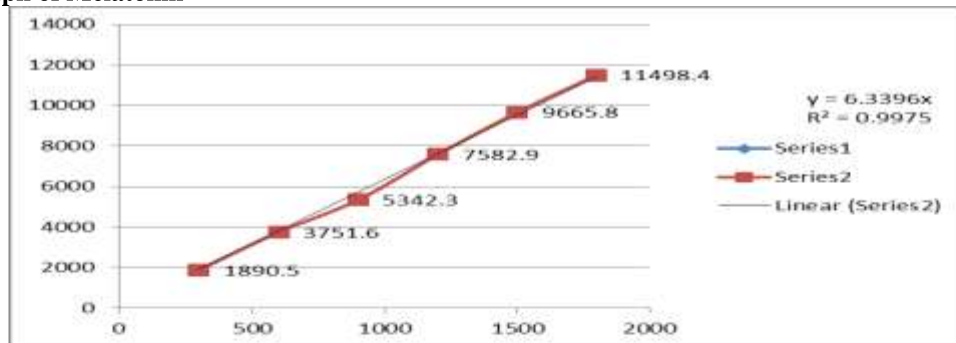
Table. 5: Assay result of marketed formulation.

Formulation	Drug	Amount taken	Amount found (ng/spot)	Labeled claim (mg) (ng/spot)	Amount found per tablet(ng)	% Label claim±SD
Strensil	ALP	200	200.07	0.50	0. 49	99.8± 0.5
	MELA	1200	1188.837	3	3.08	98.7±1.36

Linearity graph of Alprazolam



Linearity graph of Melatonin



Phtograph ofdeveloped HPTLC plate of ALP and MELA at 266nm

CONCLUSION

Thus, the objective of project work was development and comparison of analytical method of ALP and MELA in their combined dosage form. The developed and validated HPTLC method for ALP and MELA was found to be simple, specific, and cost effective and can be routinely applied for analysis of ALP and MELA in their combined dosage form. We can say that HPTLC method is more sensitive giving precise results (interday, intraday) for both drugs and also HPTLC method is more sensitive in terms of LOD and LOQ. It also requires least solvents for analysis. The proposed method has the advantages of simplicity and convenience for the separation and quantitation of ALP and MELA in combination and can be used for the assay of their dosage form. Also, the low solvent consumption and short analytical run time lead to environmentally friendly chromatographic procedures. The additives usually present in the pharmaceutical formulations of the assayed analytes did not interfere with determination of ALP and MELA. The method can be used for the routine simultaneous analysis of ALP and MELA in pharmaceutical preparations.

DISCLOSURE

The usage of this trade mark symbol or company name is for proving the genuinity of the work and not for any another purpose. The authors of the paper do not have any financial relation with the commercial identity mentioned in the paper.

CONFLICT OF INTERESTS

The authors have no conflict of interests or no financial gains in mentioning the company names or trademarks.

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