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Validated HPLC and HPTLC Methods for the Determination of Agomelatine in Bulk and Tablets

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Abstract

The present study represents validated simple, rapid and reliable chromatographic methods for analysis of the antidepressant drug agomelatine (AGO) in bulk powder and tablets. Method I involved application of RP-HPLC with diode array detection where Agilent Zorbax Eclipse-C18 column (4.6 × 150 mm, 5 µm) was used as stationary phase at ambient temperature (25±5). The mobile phase was composed of phosphate buffer (0.05 M, pH 3) and acetonitrile in the ratio 60:40 (v/v) and was pumped isocratically at 1 mL/min. In method II, HPTLC plates (20 × 10 cm, aluminum plates with 200-µm thickness precoated with silica gel 60 F254) was used as stationary phase with mobile phase composed of chloroform: methanol (9.3: 0.7, v/v). Detection was carried out at 230 nm in both methods. The structurally related melatonin was used as internal standard (IS) in both methods. The developed methods were validated according to International Council for Harmonization (ICH) guidelines with respect to linearity, ranges, accuracy, precision, robustness and limits of detection and quantitation. Linearity ranges were 0.5-3 µg/mL and 25-200 ng/spot in method I and method II respectively. Limits of detection and quantitation were 0.081 and 0.25 µg/mL for method I and 4.65 and 14.11 ng/spot for method II, respectively. Intra and inter-day precision were verified by the RSD% values which were less than 2%. The methods were implemented for assay of AGO tablet dosage form with no observable interferences.

Keywords: Agomelatine; HPLC-DAD; HPTLC; Melatonin; Internatal standard.

1. Introduction

Depression is a disturbance of mood that is distinguishable from the usual mood fluctuations of everyday life. The core features of depression are low mood, anhedonia (the loss of interest in former pleasures or activities), lethargy, anorexia, weight loss, insomnia, early morning waking, and psychomotor retardation. Depressive episodes generally last for at least 2 weeks and are classified according to severity as mild, moderate, severe, or severe with psychosis. The etiology of depression is unknown but it may be representing interaction between psychological and biochemical mechanisms rather than any single factor [1]. Guidelines have been issued nationally evidence-based recommendations for the treatment of depression. Patients with moderate to severe depression should be treated with an antidepressant [2], in addition, psychological treatments (particularly cognitive behavioral therapy) are also effective [2].

The studied drug, Agomelatine (AGO) (Figure 1) is N-[2-(7-methoxy-1-naphthyl) ethyl]acetamide [1]. AGO is an agonist at melatonergic MT1 and MT2 receptors and an antagonist at 5-HT2C receptors. It

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has antidepressant actions and is used orally in the treatment of depression in doses of 25 to 50 mg given daily at bedtime [1]. The reports found in the literature for AGO determination focused on chromatographic methods, among these, RP-HPLC was the predominant technique. The reported methods dealt with the determination of AGO in bulk form and pharmaceutical dosage forms by HPLC with UV detection at 230 nm and for determination of its potential impurities [3], and in presence of some antidepressant drugs and vitamin B12 in pharmaceutical products and urine sample [4] . In addition, LC-MS/MS methods for estimation of AGO and other antidepressant drugs in human plasma were also described [5, 6]. Besides, UHPLC-DAD-MS/MS was employed to study photostability of AGO [7]. Moreover, capillary liquid chromatography-mass spectrometry (CLC-MS) was also used for quantification of AGO in blood [8]. Stability-indicating HPLC and HPTLC methods were described for determination of AGO and its degradation products [9, 10]. Furthermore, ultra-high performance super critical fluid chromatography (UHPSFC) and ultra-high performance liquid chromatography (UHPLC) methods were employed for determination of AGO and its impurities [11]. Additionally, evaluation of AGO stability under different stress conditions using HPLC with fluorescence detection (HPLC-FD) was described [12]. Moreover, Gas chromatography–mass spectrometry (GC-MS) was employed for assay of AGO and other antipsychotics in blood [13].

UV Spectrophotometry was one of the tools applied for determination of AGO at 299 nm in bulk and dosage form [14]. Furthermore, a spectrofluorimetric method was developed for AGO determination in commercial tablets based on measuring native fluorescence of AGO at wavelengths of excitation and emission 230 and 360 nm respectively [15]. In addition, AGO is determined based on the formation of a charge transfer complex with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) and 7,7,8,8-tetracyanoquinodimethane (TCNQ) and measurement of the formed fluorophore at wavelengths of excitation and emission λex 365 / λem 475 nm and λex 250 / λem 304 nm respectively [15]. Finally, electrochemical analysis method was also utilized for determination of AGO using composite electrode comprising modified glassy carbon electrode and CeO2 nanoparticles [16]. This study presents validated simple, rapid and reliable HPLC and HPTLC methods for determination of AGO in dosage form by using the structurally related Melatonin as an internal standard (IS) in both methods. The chemical structure of AGO is very similar to that of MEL where MEL has an indole ring system, AGO has a naphthalene bioisostere instead [17] (Figure 1).

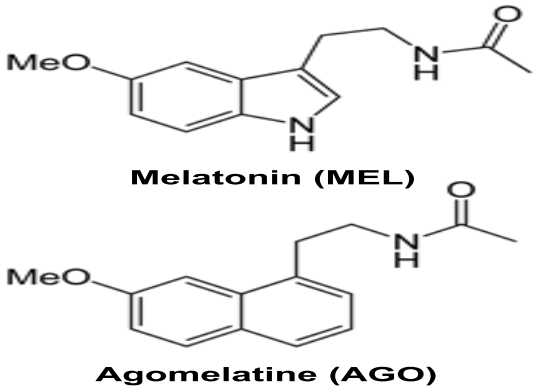


Figure 1: Structures of Agomelatine (AGO) and IS (MEL)

2. Experimental

2.1. Instrumentation:

In method I, separation was performed using HPLC-DAD Agilent 1200 series (auto-injector, quaternary pump, vacuum degasser and diode array and multiple wavelength detector G1315 C/D and G1365 C/D) connected to a computer loaded with Agilent ChemStation Software (Agilent Technologies, Santa Clara, CA, USA).

In method II, HPTLC plates (20×10 cm, aluminum plates with 200-µm thickness precoated with silica gel 60 F254) E. Merck (Darmstadt, Germany). The samples were applied to the plates using a 100-µL Camag microsyringe (Hamilton, Bonaduz, Switzerland) in the form of bands using a Camag Linomat IV (Switzerland) applicator. The slit dimension was kept at 6×0.2 mm and a 20-mm s-1 scanning speed was employed. Ascending development of the mobile phase was carried out in 20×10 cm twin trough glass chamber (Camag, Switzerland). The optimized chamber saturation time for the mobile phase was 30 min at room temperature (25◦C±2). Densitometric scanning was performed on a Camag TLC scanner III operated in the reflectance-absorbance mode and controlled by CATS software (V 3.15, Camag). The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm. The pH values of solutions were adjusted with a pH-meter Model pH211 (Hanna Instruments, USA). Calibration was done using standard buffers of pH 4 and 7 at room temperature.

2.2. Materials and reagents:

Agomelatine (AGO) 99.9% and melatonin 99.9% (MEL) were kindly supplied by National Organization for Drug Control and Research (Cairo, Egypt). All chemicals used were of analytical reagents grade. Acetonitrile HPLC grade was obtained from Research-Lab Fine Chem, Mumbai, India. Methanol was obtained from Alpha Chemika, India. Chloroform and disodium hydrogen phosphate were purchased from El-Nasr Chemical Co., Egypt. Orthophosphoric acid (85%) and glacial acetic acid (99.5%) were provided by S.D. Fine Chemicals Limited, India, and high purity distilled water was used.

2.3. Pharmaceutical preparations:

Inspago® tablets labeled to contain 25 mg AGO per tablet (batch no. 2650; manufactured by InspirePharma, Cairo, Egypt) were obtained from the local market.

2.4. Standard stock solutions:

An accurately weighed amount (10 mg) of AGO was transferred into a 100 mL volumetric flask and dissolved in about 60 mL methanol. The resulting solution was diluted to the mark with methanol to provide a stock standard solution containing 100 µg/mL. An accurately weighed amount (50 mg) of MEL (IS) was transferred into a 100 mL volumetric flask and dissolved in about 60 mL methanol. The resulting solution was diluted to the mark with methanol to provide a stock standard solution containing 500 µg/mL. Both solutions were utilized for methods I and II. The prepared stock solutions were stored refrigerated at 4 °C.

3. General procedure and construction of calibration curve

For method I (HPLC-DAD), a mobile phase system consisting of 0.05 M phosphate buffer solution (pH 3) and acetonitrile (60:40 by volume) was used. Phosphate buffer solution was prepared by dissolving 3.54 g of disodium hydrogen phosphate in 100 mL distilled water and pH was adjusted to 3 by diluted orthophosphoric acid solution and then the volume completed to 1000 mL by distilled water. The resulting phosphate buffer was filtered through a 0.45 µm membrane filter. The separation was achieved with isocratic elution at flow rate 1 mL/min. The eluent was monitored by the diode array detector (DAD) from 200 to 400 nm and chromatograms were extracted at the wavelength 230 nm for AGO and IS. All determinations were performed at 25℃. Accurate volumes (50 - 350 µL) of AGO stock solution were transferred into a set of 10 mL volumetric flasks followed by addition of 100 µL of MEL (IS) in each flask and volume was completed with water to give the final concentrations within the range 0.5 – 3 µg/mL of AGO and 5 µg/mL of MEL (IS) . Triplicate 50 µL injections were made for each concentration and chromatographed under the previously described HPLC conditions. The ratios of AGO to IS peak areas were plotted against the corresponding concentrations of AGO to obtain the calibration graph.

For method II (HPTLC), a 30 mL volume of the solvent mixture of chloroform and methanol (93: 7) was used as mobile phase. The optimized chamber saturation time for the mobile phase was 30 min at room temperature. Densitometry scanning was performed at 230 nm. Accurate volumes (250 - 2000 µL) of AGO standard stock solution were transferred into a set of 10 mL volumetric flasks followed by addition of 150 µL of MEL (IS) and volume was completed with methanol to give the final concentrations 25-200 ng/spot of AGO and 500ng/spot of MEL (IS). Triplicate 10-µL injections for the prepared working standard solutions were applied to the plates in the form of compact bands (5 mm width, 6 mm apart and 15 mm from the bottom of the plate) and chromatographed under the above-mentioned conditions. The ratios of AGO to MEL peak areas were plotted against the corresponding concentrations of AGO to obtain the calibration graph.

4. Tablet Extraction Procedure:

The developed HPLC and HPTLC methods were applied for analysis of AGO in Inspago® tablets. Ten tablets were weighed and finely powdered. An accurately weighed portion of the powder equivalent to 10 mg AGO was extracted into 50 mL methanol with the aid of shaking for 10 min then filtered through Whatman filter paper into a 100 mL volumetric flask. The residue was washed with 2 × 10 mL portions of methanol and washings were added to the filtrate, then the solution was completed to the mark with methanol to obtain 0.1 mg/mL (100 µg/mL) AGO. The prepared sample stock solution

was stored refrigerated at 4 °C. Accurate volumes of the prepared sample stock solution giving final concentrations within the previously mentioned linearity ranges were transferred into sets of 10 mL volumetric flasks, spiked with the same previously mentioned volumes of IS and the general procedures were then followed.

5. Results and discussion

The experimental parameters affecting the chromatographic separation of AGO and IS were studied and optimized. One-by-one sequential strategy is applied for optimizing each experimental variable. Variables are optimized by changing each in turn, while keeping all others constant to obtain the highest number of theoretical plates and good resolution.

5.1. Optimization and development of method I (HPLC-DAD):

An isocratic elution chromatographic method coupled with diode array detection was developed to provide a suitable procedure for the routine quality control analysis of AGO using MEL as IS. The most important aspects in HPLC method development are the achievement of high number of theatrical plates and sufficient resolution between peaks with acceptable peak symmetry in a reasonable analysis time. To achieve these goals, several experiments were carried out in order to optimize both the stationary and mobile phases. For optimization of the stationary phase, Zorbax Eclipse-C18 column (150 × 4.6 mm) was tested and it offered clear separation between the eluting symmetric peaks of AGO and MEL with relatively shorter retention times, hence; it became the column of choice for this study.

Several mobile phases were evaluated using various proportions of phosphate buffer and organic modifiers such as methanol and acetonitrile. Methanol was tried as an organic modifier where peaks suffered from increased retention times and chromatograms showed broad peaks with decreasing number of theoretical plates. Acetonitrile was then tested and it showed sharper peaks accompanied with higher number of theoretical plates and less retention times as shown in Table 1. The percentage of acetonitrile was investigated using different ratios within the range of 30-50%. Ratios of 30% and 35% of acetonitrile resulted in delayed retention times and the peaks were relatively broad. Alternatively, ratio of 50% acetonitrile led to early elution of both peaks to the extent that an overlay between solvent and IS peaks was noticed. Consequently, a ratio of 40% acetonitrile provided reasonable retention times without any overlapping between peaks. Hence, the percentage of 40% acetonitrile was found optimum as it presented the highest number of theoretical plates (Table 1). On the other hand, effect of pH of phosphate buffer was studied within the range of 2-5 (Table 1). At pH 2, overlapping was observed between the early eluting IS and solvent peak. In other respects, significant increase in peak widths were noticed at pH 4 and 5, and this led to substantial decrease in peaks symmetry and column efficiency expressed as number of plates. Hence, the optimum pH was found to be 3 as it presented the highest number of theoretical plates, symmetric peaks and reasonable retention times for both AGO and MEL. Furthermore, the effect of the flow rate on the current separation was studied. The flow rate was changed over the 0.8 to 1.2 mL/min and it was clear that the best flow rate of mobile phase was found to be 1 mL/min which was optimal for good separation as it gave the highest number of theoretical plates with reasonable retention times. Consequently, it became clear that the isocratic elution with ratio of mobile phase (40:60, v,v%) acetonitrile: phosphate buffer (0.05 M, pH 3) was the best for this method. Flow rate was kept constant at 1 mL/min all-over the run and column temperature was adjusted at 25 ^{o}C.

Due to structural similarity, AGO and IS exhibited broad absorption bands over the range 200 - 300 nm with maximum absorption at 230 nm for both AGO and IS (Figure 2). Therefore, the wavelength 230 nm was found suitable to record all chromatograms in this study and to measure peak areas for both AGO and IS. The previously described chromatographic conditions showed excellent separation of both peaks at retention times 2.33 and 6.04 min for MEL (IS) and AGO, respectively. Figure 3 shows a typical chromatogram for separation AGO and MEL (IS). Retention times, capacity factors, theoretical plates, resolution values and other system suitability parameters were calculated and they were found acceptable as shown in (Table 2).

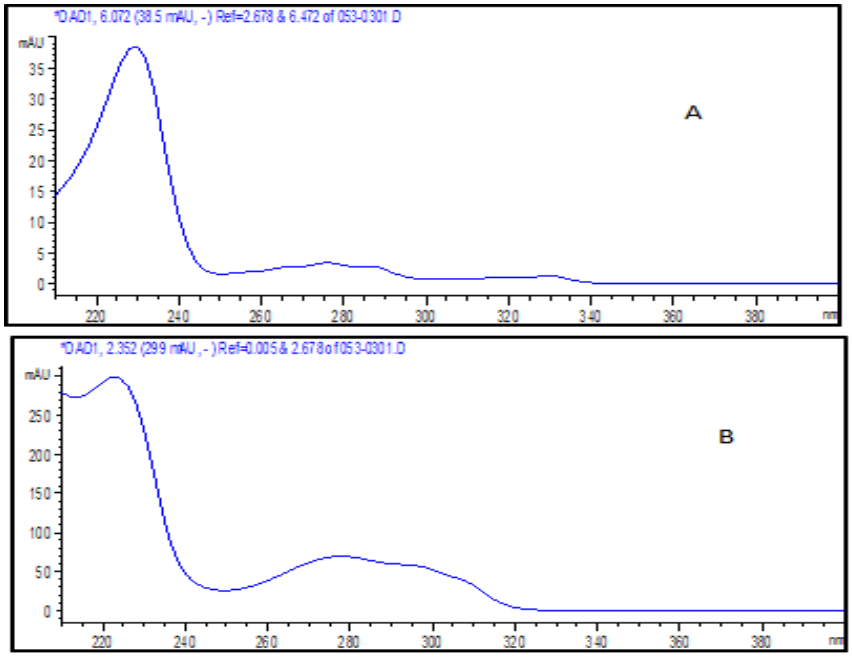


Figure 2: UV absorption spectra of AGO (A) and MEL (IS) (B) obtained from DAD.

Table 1: Effect of different experimental parameters on the column efficiency (number of theoretical plates), capacity factor and resolution for optimization of method I

Experimental factor		Capacity factor (k′)		Theoretical Plates (N)		Resolution (R_s)	
		MEL	AGO	MEL	AGO	MEL	AGO
Acetonitrile Concentration	30%	1.06	3.90	3393	5126	13.57	
	35%	0.92	3.73	3546	5244	14.71	
	40%	0.76	3.58	3860	5389	15.75	
	50 %	0.53	3.03	1542	5112	12.73	
Type of organic modifier	ACN	0.76	3.59	3850	5382	15.71	
	Methanol	0.93	3.62	1708	3600	12.12	
Phosphate buffer pH	2	0.29	3.06	1184	5186	13.78	
	3	0.77	3.61	3543	5234	14.75	
	4	0.59	2.91	2286	4139	13.20	
	5	0.73	2.09	2208	3912	9.85	
Flow rate (mL/min)	0.8	0.98	3.89	3136	4003	13.12	
	1	0.76	3.60	3844	5377	15.73	
	1.2	0.54	3.40	2916	4868	15.84	

Table 2: System suitability parameters for the separated compounds in the proposed HPLC-DAD method.

Compound	t_R ± SD (min)	Capacity factor (k′)	Theoretical Plates (N)	Symmetry	Selectivity (α)	Resolution (R_s)
MEL	2.33 ± 0.010	0.76 ± 0.014	3844 ± 0.02	0.87		
AGO	6.05 ± 0.017	3.59 ± 0.022	5377 ± 0.03	0.95	2.51	21.93

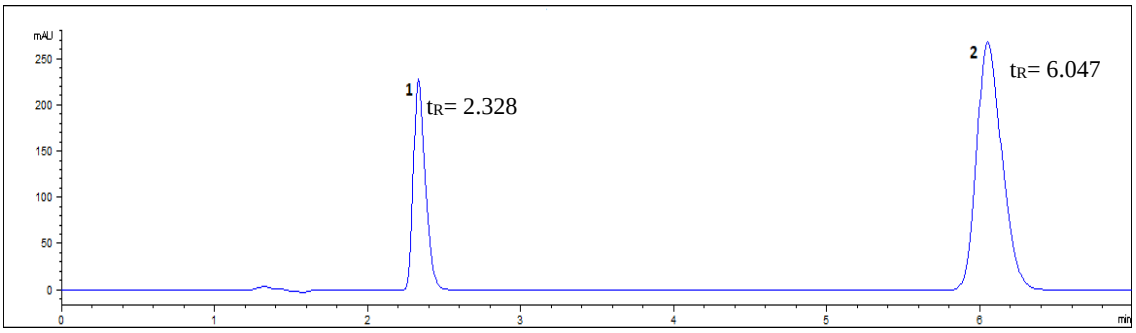


Figure 3: HPLC chromatogram of 5 μg/mL of IS (1) and 3 μg/mL of AGO (2) at 230 nm

5.2. Optimization and development of method II (HPTLC)

For HPTLC analysis (method II), various practical conditions were studied to achieve optimum separation of AGO and IS. Simple mobile phase combinations composed of chloroform and methanol with different ratios were tried. First, ratio of (5:5, v/v) was tried and this led to migration of both AGO and MEL (IS) with same high retardation factors without proper separation between them, besides the spots were adjacent to the solvent front. To achieve acceptable separation between spots, the polarity of mobile phase was reduced by decreasing volume of methanol in further trials. A ratio of chloroform and methanol (7:3, v/v) was tested and resulted in slightly better separation between spots. Additionally, the retardation factors were also high which were 0.81 and 0.86 for AGO and IS respectively, and the spots remained nearby solvent front. Adequate separation between spots was attained by further reduction of methanol percentage in the mobile phase to be only 10 % with 90% chloroform. Although both spots were adequately separated at Rf values 0.64 and 0.83 for IS and AGO, respectively, however, AGO spot travelled long distance to reach Rf close to the solvent front which implied that complete resolution between spots needed longer time. Consequently, further decrease in methanol percentage in the mobile phase was required to ensure both complete separation between spots and reasonable average retardation factors. Finally, using chloroform-methanol (9.3: 0.7, v/v) mixture achieved the best separation of AGO and MEL (IS) with appropriate retardation factors equal 0.39 and 0.63 For IS and AGO respectively. Likewise method I, AGO and IS exhibited broad absorption bands over the range 200 - 300 nm with maximum absorption at 230 nm for both AGO and IS (Figure 4). Consequently, all chromatograms and peaks areas were recorded at 230 nm. System suitability parameters were calculated as an integral part of the chromatographic procedure. Insignificant peak tailing is an advantage which results in less interference between peaks with more accurate calculation of the peak areas. The tailing factors were 0.88 and 1.20 for MEL (IS) and AGO, respectively; indicating acceptable peak asymmetry. Concerning sample loading, solutions were applied to the marked start edge of the TLC plate at a height of 15 mm from the lower edge of the plate using the specified TLC CAMAG linomat syringe. Sample injection volume was tried to be 20 μL and 10 μL, and it appeared that optimum chromatograms were achieved by injection volume of 10 μL, as larger injection volumes led to increased peak widths and consequently less resolution. Spotting was performed in the form of bands of 5-mm width and the spots were kept at a constant distance of 6 mm from each other to avoid edge effect. On applying these chromatographic conditions, excellent separation between AGO and IS with accepted Rf values was attained; 0.39 and 0.63 for MEL (IS) and AGO, respectively. Furthermore, sufficient resolution and symmetric peak shape were also accomplished. Figure 5 shows a typical chromatogram for separation between AGO and IS.

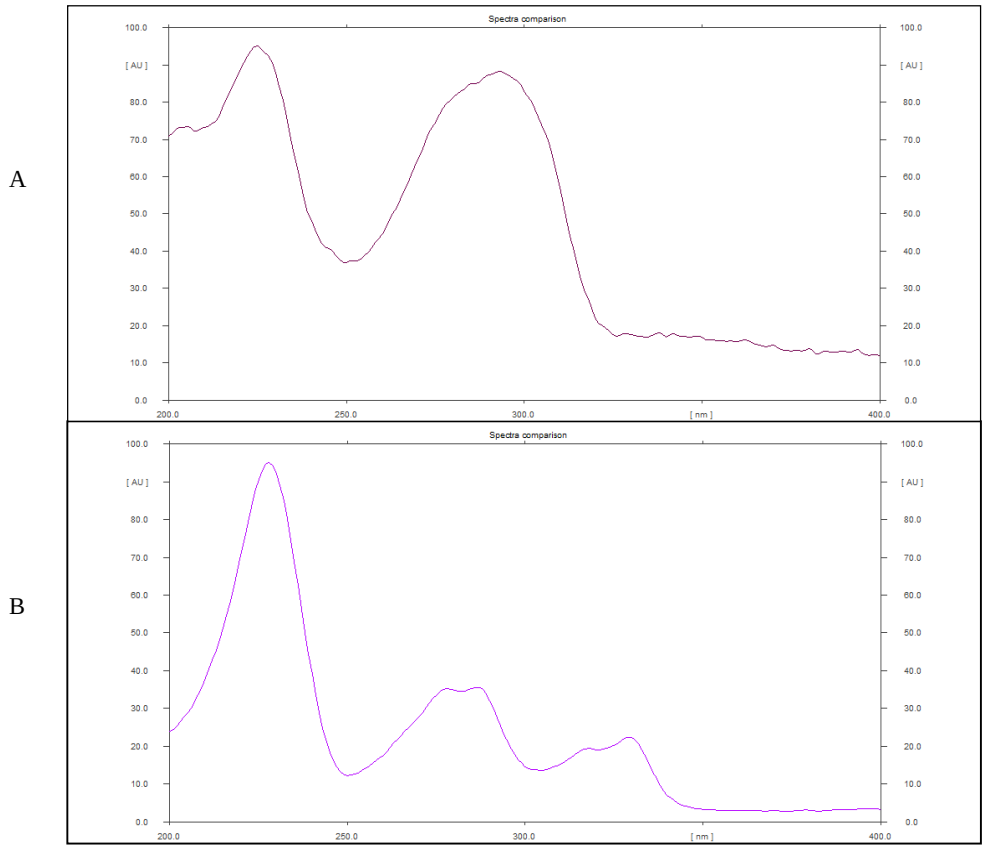


Figure 4: UV absorption spectra of AGO (A) and MEL (IS) (B) obtained by HPTLC scanner

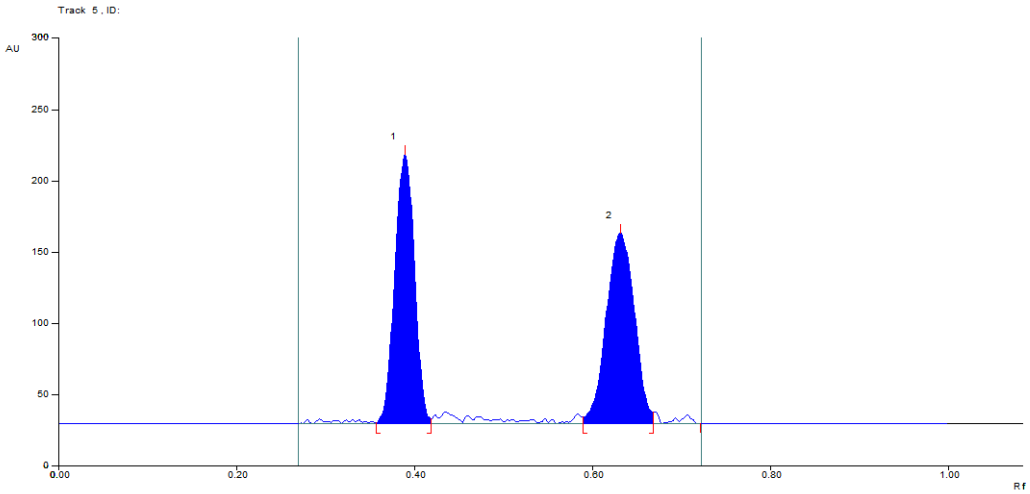


Figure 5: HPTLC chromatogram of 500 ng/spot of MEL (1) and 200 ng/spot of AGO (2) at 230 nm.

6. Analytical validation

Both proposed HPLC and HPTLC methods were validated as per the International Council for Harmonization (ICH) guidelines on validation of analytical procedures (Q2R1)[18].

6.1. Linearity and concentration ranges

The linearity of the proposed procedures was evaluated by analyzing a series of different concentrations for AGO. According to ICH guidelines, at least five concentrations must be used. The linear regression equations were generated by the

least-squares treatment of the peak area ratios versus the corresponding concentrations (n = 6). Regression analysis for the calibration curves showed good linear relationships over the concentration ranges of 0.5-3 µg/mL and 25-200 ng/spot for method I and II respectively. Good linearity was indicated from the correlation coefficient values > 0.999 (r = 0.9997) for both methods with RSD% of slope values (Sb %) less than 2% (1.27 and 1.21) for method I and method II respectively. Table 3 present the performance data and statistical parameters including linear regression equations, concentration ranges, correlation coefficients, standard deviations of the intercept (S_a), slope (S_b) and standard deviations of residuals ($S_{y/x}$).

6.2. Limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection is the lowest concentration of the analyte that can be detected but not necessarily quantitated under the applied experimental conditions while the limit of quantitation is the lowest concentration that can be determined with acceptable precision and accuracy. The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated according to the ICH guidelines. The limit of detection (LOD) is defined as the concentration of the analyte which has a signal-to-noise ratio of 3:1. For the limit of quantitation (LOQ), the ratio considered is 10:1. They are given in Table 3. The LOD values of AGO are 0.08 µg/mL and 4.65 ng/spot for method I and II respectively, while the LOQ are 0.25 µg/mL and 14.11 ng/spot in method I and II respectively. Both LOD and LOQ values confirm the sensitivity of the proposed HPLC and HPTLC procedures.

6.3. Accuracy and precision

The accuracy and within-day (intra-day) precision for the proposed methods were examined at three concentration levels within the studied linearity ranges (0.5, 2 and 3 µg/mL for method I; 50, 100 and 200 ng/spot for method II) using three replicate determinations for each concentration within one day. Similarly, the accuracy and between-day (inter-day) precision were tested by analyzing the same three concentrations for each compound using three replicate determinations repeated on three consecutive days. Recoveries were calculated using the corresponding regression equations and they were satisfactory. The percentage relative standard deviation (RSD %) and percentage relative error (E_r %) did not exceed 2.0 % proving the high precision and accuracy of the proposed methods for the estimation of the AGO in their bulk form (Table 4).

6.4. Specificity

Method specificity can be partially demonstrated by the successful resolution of both AGO and its structurally related compound MEL. Figure 6 and 7 show typical chromatograms for separation of AGO from MEL (IS) in tablet dosage form in method I and II respectively. The peak purity of AGO and IS was assessed by comparing their spectra at peak start, apex and end positions of the peak (Figure 8 and 9) in method I and II respectively. Acceptable peak purity suggests no interference from the tablet excipients in the quantification of the AGO in sample solutions, and this can be considered another demonstration for method specificity.

6.5. Robustness

The robustness of analytical procedure is a measure of its capability to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage [18].

For method I, robustness was examined by making small changes in acetonitrile content in the mobile phase (± 2%), flow rate (± 0.2 mL/min) , working wavelength (± 2 nm) and pH of phosphate buffer solution (± 0.1) and recording the chromatograms of the mixture of AGO and IS. For method II, the parameters studied were mobile phase ratio (± 0.6 mL), mobile phase volume (± 3 mL), duration of saturation (± 10 min), time from chromatographic separation to scanning (± 10 min) and working wavelength (± 2 nm). Recording the chromatograms of a standard mixture was done after each deliberate change. These variations did not have any significant effect on retention times or retardation factors of the eluting peaks or the measured responses (peak areas) of AGO and MEL (IS). Table 5 and 6 show the effects of the studied variations on chromatographic parameters of AGO and IS peaks and peak areas in method I and II respectively. Additionally, these minor experimental changes did not affect the resolution between AGO and IS. A minimum resolution value of 1.5 is usually regarded as sufficient for the baseline separation of closely eluted peaks [19]. Resolution between two peaks was

never less than 13 for method I which implies excellent baseline separation.

6.6. Stability of solutions

The stability of AGO and MEL standard solutions as well as the sample solutions in methanol was examined. Retention times and peak areas of both drugs remained unchanged and no detectable degradation was observed during 2 days at room temperature (± 25℃) and for one week when stored refrigerated at 4 ℃.

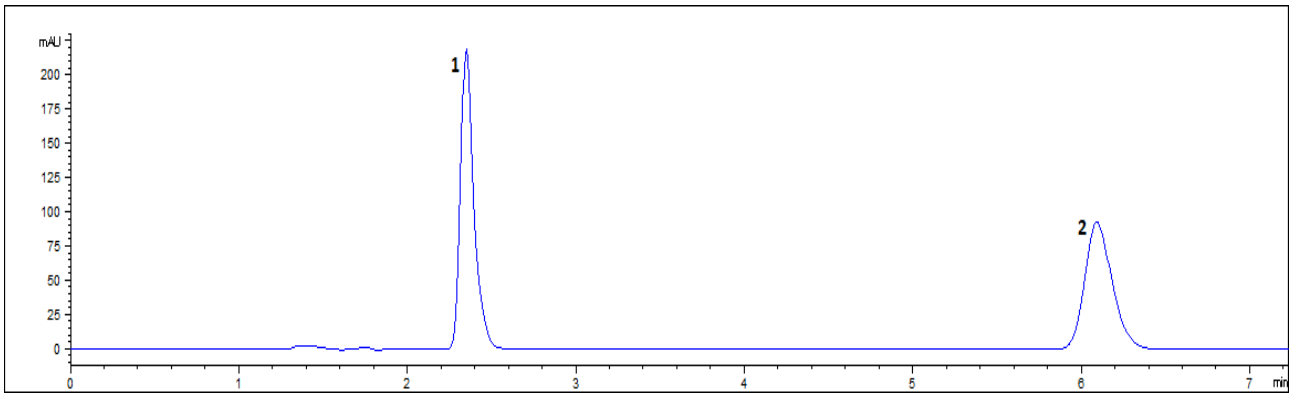


Figure 6: HPLC chromatogram at 230 nm of a 20-µL injection of a solution containing 5 µg/mL MEL and 3 µg/mL AGO obtained from Inspago® tablet.

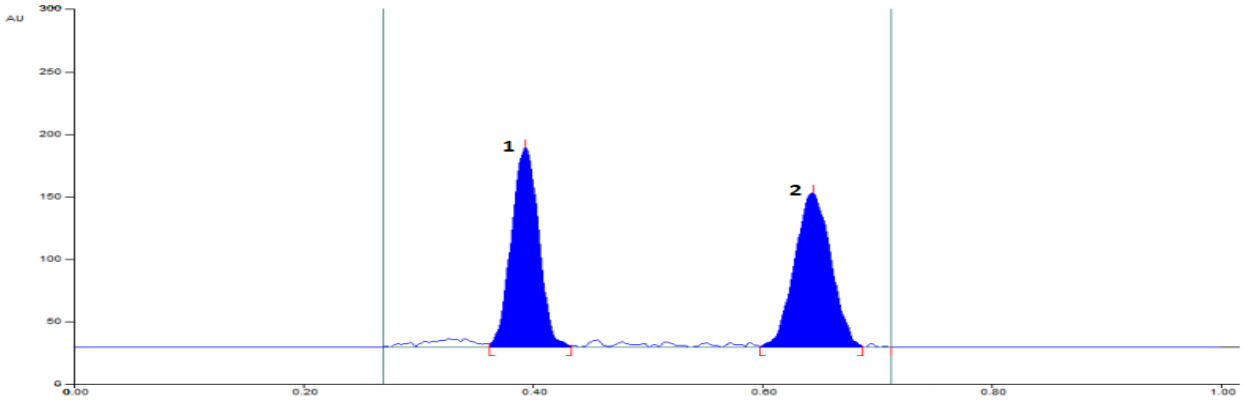


Figure 7: HPTLC chromatogram at 230 nm of a 10-µL injection of a solution containing 500 ng/spot MEL and 200 ng/spot AGO obtained from Inspago® tablet.

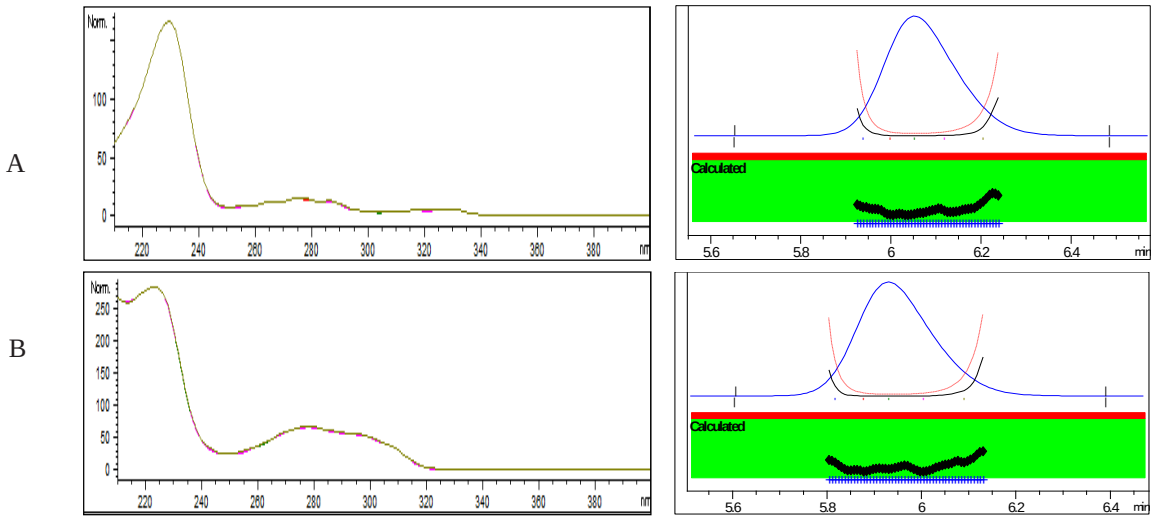
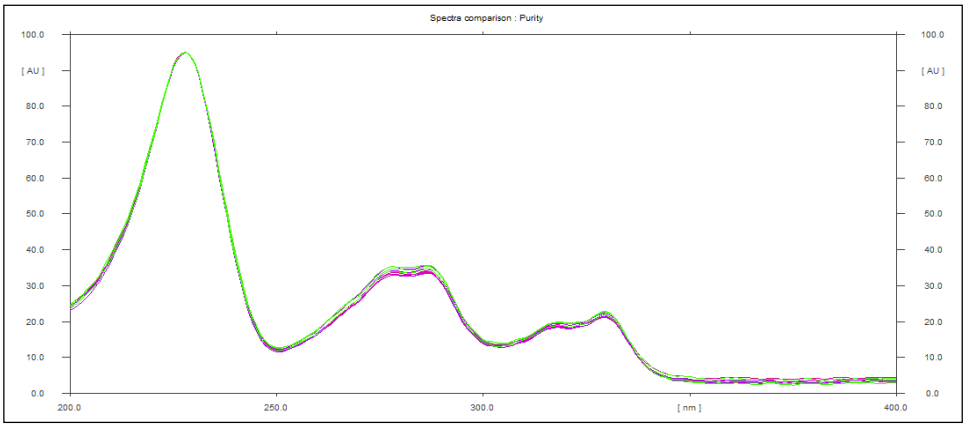


Figure 8: Purity spectra of AGO (A) and IS (B) obtained by DAD

A



B

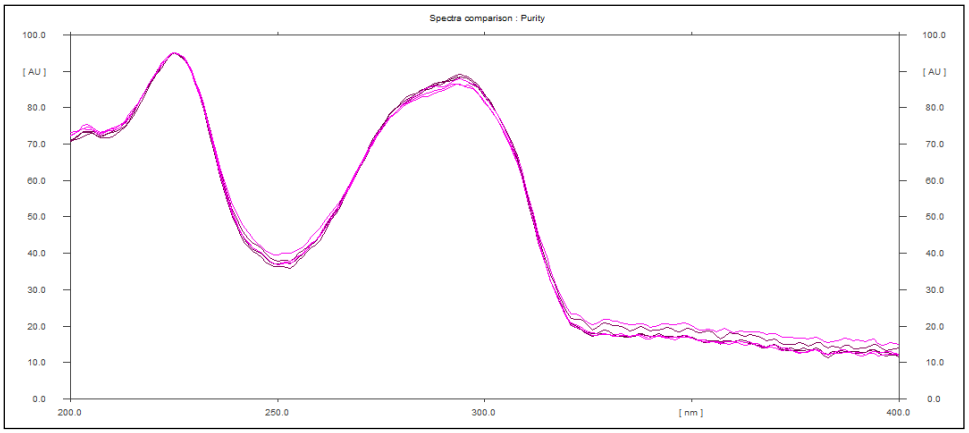


Figure 9: Purity spectra between 200 and 400 nm of AGO (A) and MEL (B) obtained by using HPTLC scanner

Table 3: Analytical parameters for the determination of AGO in HPLC-DAD and HPTLC method.

Parameter	HPLC-DAD (Method I)	HPTLC (Method II)
Concentration range (µg/mL) in method I (ng/spot) in method II	0.5 - 3	25 - 200
Intercept (a)	0.005	-0.018
S_a	0.020	0.014
Slope (b)	0.830	0.010
S_b	0.010	0.0001
RSD% of the slope	1.27	1.21
Correlation coefficient (r)	0.9997	0.9997
$S_{y/x}$	0.022	0.018
F value	6228	6798
Significance F	1.540×10^{-7}	1.290×10^{-7}
LOD (µg/mL) in method I (ng/spot) in method II	0.080	4.650
LOQ (µg/mL) in method I (ng/spot) in method II	0.250	14.110

Table 4: Precision and accuracy for the analysis of AGO in bulk form using the proposed HPLC-DAD and HPTLC methods

		Concentration (μg/mL) in method I (ng/spot) in method II	Found ± SD*	RSD (%)	E_r(%)
HPLC-DAD	Within-day	**0.5**	0.49 ± 0.004	0.82	- 2.00
		2	2.01 ± 0.020	0.99	0.50
		3	2.98 ± 0.030	1.01	- 0.67
	Between-day	**0.5**	0.50 ± 0.006	1.20	0.00
		2	2.00 ± 0.020	1.00	0.00
		3	3.03 ± 0.008	0.26	-1.00
HPTLC	Within-day	**50**	50.50 ± 0.590	1.17	1.00
		100	100.08 ± 1.180	1.18	- 0.08
		200	199.14 ± 1.850	0.93	- 0.43
	Between-day	**50**	49.78 ± 0.980	1.97	- 0.50
		100	100.25 ± 0.950	0. 95	- 0.25
		200	198.60 ± 0.650	0.33	- 0.70

*Mean ± standard deviation for three determinations.

Table 5: Evaluation of robustness of the proposed HPLC-DAD method.

Parameter	Compound	Retention time t_R± SD	Resolution (R_s ± SD)	Peak area ratio ± SD	RSD%
Acetonitrile percentage in the mobile phase ± 2%	MEL	2.33 ± 0.141			
	AGO	5.91 ± 0.860	15.41 ± 1.303	1.621 ± 0.004	0.92
Flow rate ± 0.2 mL/min	MEL	2.30 ± 0.230			
	AGO	5.77 ± 0.500	14.07 ± 4.570	1.644 ± 0.030	1.73
Working wavelength ± 2 nm	MEL	2.31			
	AGO	5.76	14.90 ± 0.012	1.573 ± 0.228	0.83
pH of phosphate buffer ± 0.1	MEL	2.32 ± 0.008			
	AGO	5.91 ± 0.043	15.61 ± 0.190	1.580 ± 0.003	0.22

Table 6: Evaluation of robustness of the proposed HPTLC-DAD method.

Parameter	Compound	Retardation factor (Rf) ± SD	Peak area ratio ± SD	RSD%
Mobile phase volume ± 3 mL	MEL	0.395 ± 0.017		
	AGO	0.627 ± 0.030	0.971 ± 0.012	1.28
Duration of saturation ± 10 min	MEL	0.476 ± 0.096		
	AGO	0.711 ± 0.116	0.929 ± 0.016	1.80
Mobile phase ratio ± 0.6 mL	MEL	0.443 ± 0.132		
	AGO	0.668 ± 0.116	0.925 ± 0.014	1.58
Working wavelength ± 2 nm	MEL	0.378 ± 0.007		
	AGO	0.626 ± 0.015	0.932 ± 0.018	1.94
Time from Chromatography to scan ± 10 min	MEL	0.375 ± 0.005		
	AGO	0.620 ± 0.010	0.932 ± 0.014	1.50

7. Application to dosage forms

The developed HPLC-DAD and HPTLC methods were applied for the assay of AGO in its commercial pharmaceutical formulations (Inspago® tablet, batch no. 2650) labeled to contain 25 mg AGO per tablet. Recovery values were calculated from similarly treated external standard solutions. The assay results revealed satisfactory accuracy and precision as indicated from % recovery, SD and RSD% values (Table 7). The good recoveries indicated the absence of any interference from commonly encountered inactive ingredients that may be present in the tablets.

Furthermore, a simple reported spectrophotometric method was adopted for the estimation of AGO in its tablets [20]. The reported method depends on measuring the absorbance of AGO in water at 233 nm [20]. The recoveries obtained from the proposed methods were statistically compared with those of the reported method using the one-way analysis of variance (Single factor ANOVA). The calculated F-value did not exceed the critical value, indicating that there were no significant differences between the proposed methods together with the reported method (Table 7). It is evident from these results that the proposed chromatographic methods are applicable to the assay of AGO in commercial tablets with a satisfactory level of accuracy and precision.

8. Conclusion

This study described two simple, selective and reliable chromatographic methods for the assay of AGO in bulk and in pharmaceutical dosage form using isocratic elution HPLC-DAD and HPTLC procedures. The structurally related melatonin was used as internal standard in both methods. Reliability was guaranteed by testing various validation parameters of the methods and the successful application to commercial tablet dosage form. In addition, there are several chromatographic methods for AGO assay [3-13]. A comparison of the suggested methods with these reported methods illustrates that the suggested procedures offer comparable sensitivity [8, 9, 11]. Besides, the proposed methods use isocratic elution instead of gradient elution used in other methods [5, 7, 11], with only two components in the mobile phase in both methods rather than complicated mobile phases which are consisted of three or even more components [4, 5, 8], and the detection was achieved at longer wavelength (230 nm) versus 207 nm which was used in a previous study [10]. Moreover, the described procedures herein involve the use of internal standard for more reproducible and accurate assay results, while some previous studies lack this advantage [4, 9, 11]. Assay results of both methods were statistically compared with those obtained from a comparison method and the results showed agreeable precision, accuracy and applicability of the proposed methods.

Table 7: Application of the proposed HPLC-DAD and HPTLC methods to the analysis of AGO in tablet dosage form

Method / Results	HPLC (method I)	HPTLC (method II)	Reported method [20]
%Found ± SD *	99.39 ± 1.731	98.97 ± 1.460	99.97 ± 1.242
RSD%	1.74	1.47	1.24
Variance (SD^2)	1.63	2.13	1.53
ANOVA (single factor)			
F 0.08184	**F critical 3.885293**		

*Mean ± standard deviation for five determinations.

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