Egypt. J. Anal.Chem, Vol. 24, PP. 1-18 (2015)

Development and Validation of a Stability-Indicating High Performance Liquid Chromatography Method for Assay of Agomelatine

S. A. Abdalla and M. S. Abdelmoneim
National Organization for Drugs Control And Research, NODCAR, Alharam – Giza, Egypt.
Corresponding Author: Tel.+201090035411: Email: ziyadomar70@yahoo.com

Summary. An isocratic stability indicative HPLC–UV method, simple, rapid, sensitive, precise and accurate was developed and validated for assay of agomelatine in dosage forms in the presence of its degradation products. The method was developed on Waters Spherisorb C_{18} (250X4.6 mm), 5µm column using mobile phase as buffer (0.1 g potassium dihydrogen phosphate buffer pH 5.0) : Acetonitrile (65:35 v/v), at a flow rate of 1.0 ml/min and the detection was carried out at 230 nm using photo-diode array detector. Retention time of agomelatine was found to be 7.53 min. The drug was subjected to acidic hydrolysis, alkali hydrolysis and oxidation, thermal and photolytic stress condition. Complete separation of degraded products was achieved from the parent compound. All degradation products were eluted in an overall analytical run time of approximately 10 min with the parent compound agomelatine. The method was linear over the concentration range of 2-10 µg/ml (r^2 = 0.998) with a limit of detection and quantitation of 0.075 and 0.23 µg/ml, respectively. The validation element investigated showed that the method has acceptable specificity, accuracy, precision and robustness as per ICH guidelines and can be used for the quality control of the raw materials as well as formulations .

Keyword: *validation, HPLC, stability indicating, agomelatine.*

Introduction

Agomelatine, Chemical structure (N-[2-(7-methoxy-1-naphthyl)ethyl] acetamide), is a novel antidepressant developed by Servier Laboratories[(1)] with molecular weight 243.301 and having the following structure.

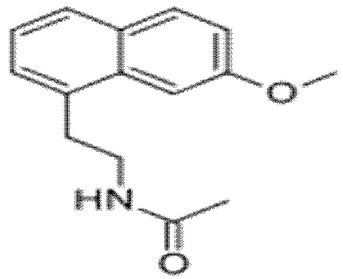


Figure 1. Chemical structure and molecular formula: $C_{15}H_{17}NO_2$

Agomelatine is a White or white alike crystal powder or supplied as a crystalline solid and it is a non-hygroscopic white or almost white powder practically insoluble in purified water and contains no asymmetric carbon atoms. Agomelatine is soluble in organic solvents such as methanol, ethanol, DMSO, and dimethyl formamide. Whereas it is sparingly soluble in aqueous buffers. For maximum solubility in aqueous buffers, agomelatine should first be dissolved in methanol and then diluted with aqueous buffer of choice[(2)]. It represents the only MT1/MT2 melatonergic receptor agonist and 5-HT2C antagonist available, shown to induce resynchronization of circadian rhythms and antidepressant action in humans[(3-5)] . Extensive clinical trials have established efficacy of agomelatine taken by major depression patients, with an improvement of sleep quality, preservation of sexual function, absence of weight gain and good tolerability[(6)]. The local product is agomela 25 mg/tablet, Debaky ZAD Company for pharmaceutical products, under registration in Egypt.

As we know, analytical methods are essential to characterize drug substances and drug products composition during all stages of pharmaceutical development. For routine analytical purpose it is always necessary to establish methods capable of analyzing large number of samples in a short time period with high accuracy and precision. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. Hence, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop new analytical methods for such drugs. These products can present challenges to the analytical chemist responsible for the development and validation of analytical methods.

Also, stress testing is an integral part of developmental strategy in developing and validating suitable analytical methods[7-8]and it is carried out under more severe condition than that of accelerated conditions. These studies provide information of drug's intrinsic stability and the total information was tabulated for different types of reactivities, viz., hydrolysis in acid, alkaline and neutral conditions, oxidation and photolysis. Regarding analysis to proposed study, some reports have been published on analysis of agomelatine [9-11] by detection with mass spectrometry or ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS–MS) . These reports involved isocratic chromatographic conditions and mass spectrometric detection in the positive ionization mode using an API-4000 system. No other methods were reported for determination of agomelatine and also, to the best of our literature survey, so far there is no published report describing validated stability indicating HPLC method for determination of agomelatine available in literature. This paper deals with force degradation of agomelatine under acidic hydrolysis, alkali hydrolysis and oxidation, thermal and photolytic stress condition and the validation of developed method for assay of agomelatine in bulk drug and in tablet dosage form.

Materials and methods

Chemicals and materials

Standard reference material of agomelatine and its impurity standard namely impurity (I), impurity (II) and impurity (III) in addition to product samples were received as gift samples from Debaky ZAD Company for pharmaceutical products. The product is presented as film-coated tablets containing 25 mg of agomelatine as active substance. Other ingredients are lactose monohydrate, maize starch, povidone, sodium starch glycolate type A, stearic acid, magnesium stearate and silica colloidal anhydrous in the core tablet and hypromellose, yellow iron oxide (E172), glycerol , macrogol 6000, and titanium dioxide (E171) and indigotine (E132). The film coated tablets are packaged in aluminium/polyvinylchloride blister pack. All reagents used were of analytical grade.

Acetonitrile and methanol (HPLC grade, far UV) was purchased from Romil Ltd. (Cambridge, UK). Potassium dihydrogen phosphate Sodium, hydroxide pellets and *ortho*-phosphoric acid (85%, w/w) were obtained from Nassar Chemicals Ltd. (Eygpt, Cairo). Membrane filters of 0.45um (Millipore) were used.

Instrumentation and chromatographic conditions

Analysis was performed on a Shimadzu LC-1100 HPLC system equipped with an online degaser DGU-20As, an Rheodyne 7725 injection valve furnished with 20 mL loop, an SPD-M20A photodiode array detector and a Class-VPsoft- ware. Separation was carried out using a Waters Spherisorb C_{18} column (250X4.6 mm i.d., 5µm pore size). The column was maintained at 27 °C throughout analysis and the UV detector was set at 230 nm. Different mobile phase were tested in order of their polarity to out the best conditions for separation of agomelatine. The selected mobile phase containing (0.1g/L) potassium dihydrogen phosphate (pH 5.0) and acetonitrile in the ratio of 65:35 gave acceptable retention time and good resolution. The mobile phase was filtered by using 0.45 µm Millipore nylon filter paper and degassed by sonication prior to use.

Preparation of standard solution

Agomelatine standard stock solution containing 100µg/ml was prepared in a 100 ml volumetric flask by dissolving 50 mg of agomelatine and then diluted to volume with methanol as a solvent. Further take 10 ml of this stock solution in 50 ml volumetric flask and make up to mark with mobile phase (Final standard solution of 100µg/ml). Aliquots of the standard stock solutions of agomelatine were transferred using A-grade pipette into 10 ml volumetric flasks and solutions were made up to the volume with mobile phase to give the final concentrations of 2-10 µg/ml.

Preparation of test solution

Twenty tablets were weighed and the average weight of tablet was determined. From these, about equivalent to ten tablets were weighed and transfer into a 250 ml volumetric flask. About 50 ml of methanol as a solvent was added and sonicated for a minimum 15 min. with intermittent shaking. Then content was brought back to room temperature and diluted to volume with the same solvent. The sample was filtered through 0.45µm nylon

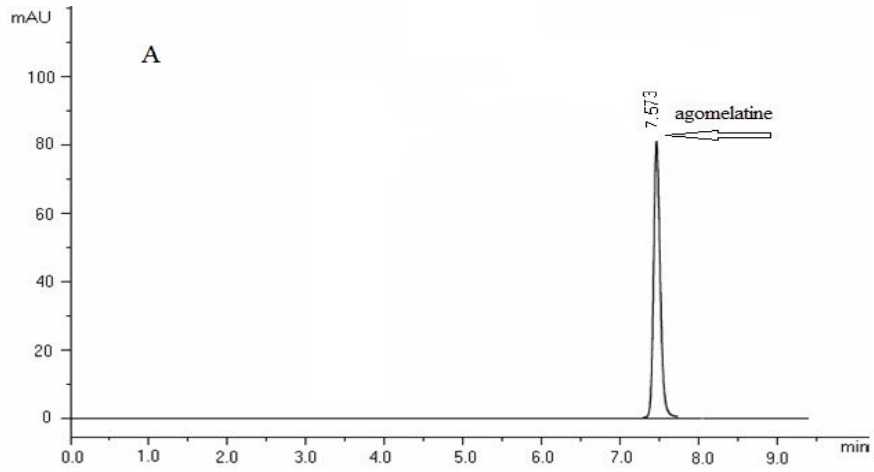
syringe filter. Further take 10 ml of this stock solution in 100 ml of volumetric flask and make up to mark with mobile phase. The final concentration obtained was 100 µg/ml of agomelatine. Suitable aliquots of the filtered solution was added to a volumetric flask and made up to the volume with mobile phase to yield the concentrations of 4, 6 and 8 µg/ml. A 20 µl volume of each sample solution was injected into HPLC, three times, under the conditions described above. The peak areas were measured at 230 nm and concentrations in the samples were determined by comparing the area of sample with that of the standard.

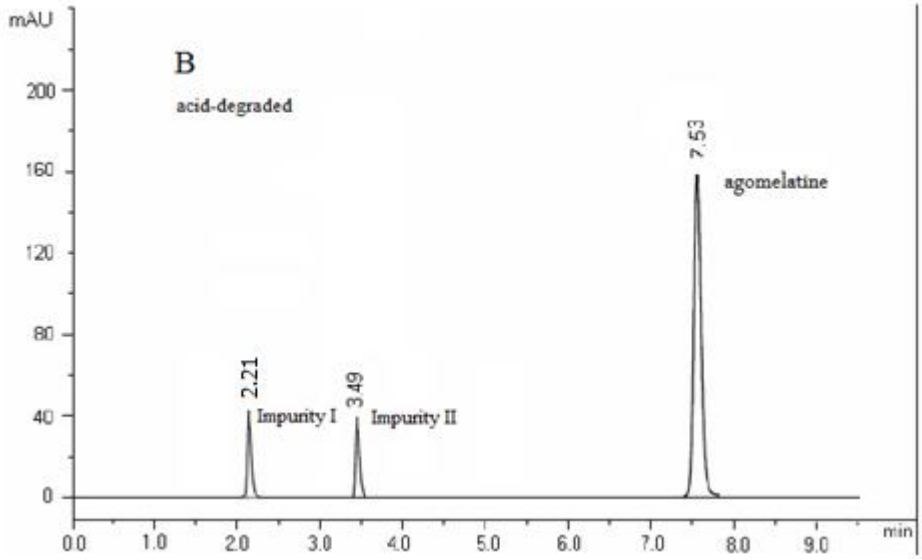
Degradation studies of Agomelatine

In order to determine whether the analytical method and assay were stability-indicating, agomelatine pure drug was stressed under various conditions to conduct forced degradation studies. As agomelatine was insoluble in water, methanol was used to dissolve the drug and then volume was made up with the same solvent. A stock solution of 100 µg/ml was prepared by dissolving 10 mg of agomelatine in 10 ml of methanol and volume was made up to 100 ml with the same solvent. This solution was used for forced degradation studies to evalute the stability indicating property and specificity of proposed method. In all degradation studies the average peak area of standard agomelatine and degradation sample after application (20 µg/ml for HPLC) of six replicates were obtained.

Oxidation, acid and alkali degradation.

To three tubes separately, each one containing 2 ml of stock solution, 2 ml of 1% hydrogen peroxide was added to the first one, 2 ml of 0.01 N hydrochloric acid to the second and 2 ml of 0.01 N sodium hydroxide to third respectively. The solutions were kept for 30 min at room temperature. For HPLC study, the resultant solutions were diluted to obtain 20 µg/ml solution and 20 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.


mAU
A
100
80
60
40
20
0
7.573
agomelatine
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
min


mAU
B
acid-degraded
200
160
120
80
40
0
2.21
Impurity I
3.49
Impurity II
7.53
agomelatine
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
min

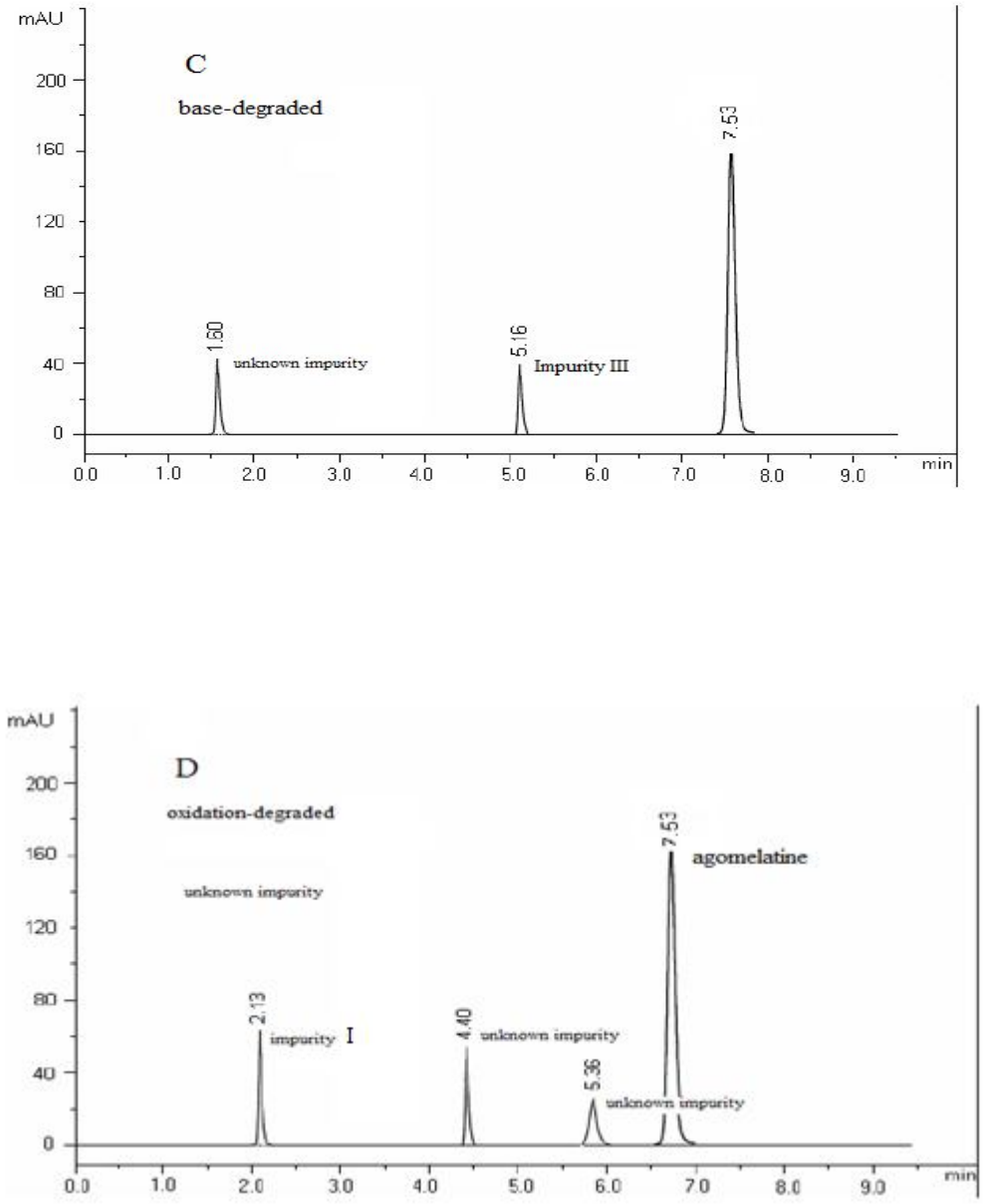


Figure 2. (A) typical chromatogram of agomelatine (6 µg/ml), (B) chromatogram of acid-degraded, (C) chromatogram of base-degraded and (D) chromatogram of oxidation-degraded.

Photochemical degradation product

The photochemical stability of the drug was also studied by exposing the stock solution (1 mg/ml) to UV light for 5 days on a wooden plank and kept on terrace. For HPLC study, the resultant solution was diluted to obtain 20 µg/ml solutions and 20 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

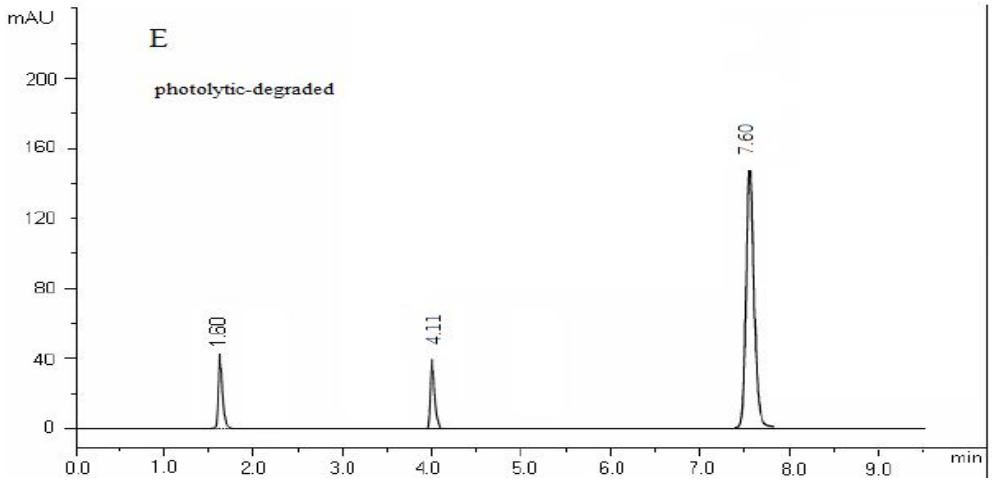


Figure 3. (E) typical chromatogram of photolytic-degraded.

Detection of the known and unknown impurities

The related impurities were determined by injecting higher concentrations of the drug and compared with standard known impurity to detect and quantify known and unknown ones. Standard solution and sample solution were applied on chromatography system and the chromatograms were run to assess stability indicating.

Results and Discussion

Chromatographic Conditions

The HPLC procedure was optimized with a view to develop a stability indicating assay method. Optimum chromatographic conditions were obtained after running different

mobile phases with a reversed phase Waters Spherisorb C_{18} column (250X4.6 mm i.d., 5µm pore size). Buffer solution with acetonitrile in the ratio mentioned before was preferred as a mobile phase because its use resulted in improved separation. Many different isocratic systems of mobile phases were tried to achieve the best separation of peaks. Initially (methanol and water) and (acetonitrile and water) in different ratios were tried. It was found that (acetonitrile and water) system gives good results than (methanol and water) as the drug was more soluble in acetonitrile than methanol due to the high dielectric constant for acetonitrile. But, Acetonitrile: water in the ratio of 70:30 was not able to give good peak symmetry with acceptable retention time. An attempt to improve peak symmetry was made by adding phosphate buffer to the mobile phase. The presence of phosphate buffer in mobile phase resulted in excellent overall chromatography with appropriate peak symmetry and complete base line resolution. Finally the mobile phase consisting of phosphate buffer and acetonitrile (65:35 v/v) with pH 5.0 adjusted with phosphoric acid was selected for validation purpose and stability studies. Selecting 230 nm as the detection wavelength resulted in an acceptable response and enable the detection of compound used in this study. The temperature of column was maintained at 27 °C throughout analysis. The modalities adopted in experimentation were successfully validated as per analytical procedures laid down in routine.

Method validation

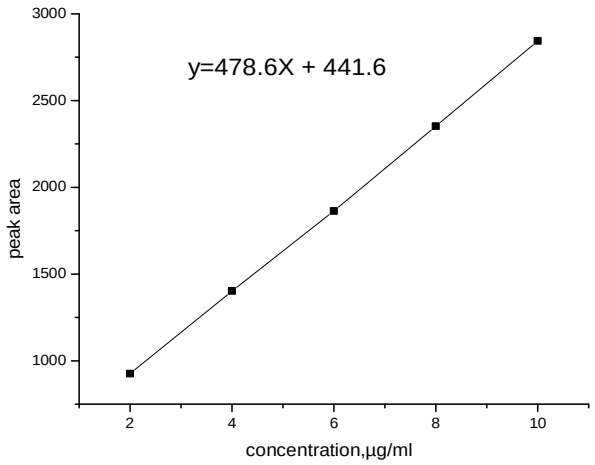
Validation of an analytical method is the process by which it is established, by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical applications whereas method validation is the process of proving that an analytical method is acceptable for its intended purpose.[(12)] So, the method was validated according to ICH[(13)] and guidelines[(14)] . The validation parameters addressed were specificity, linearity, limits of detection, precision, accuracy, robustness and quantification and the stability of agomelatine.

Specificity

The evaluation of the specificity of the method was determined against placebo. The interference of the excipients of the claimed placebo present in pharmaceutical dosage form was derived from placebo solution. Further the specificity of the method toward the drug was established by means of checking the interference of the degradation products in the drug quantification for assay during the forced degradation study The results of specificity studies indicated no interference from excipients, impurities and degraded products due to various stress conditions and assured that the peak response was due to a single component only. The method was validated with respect to parameters including linearity, limit detection (LOD), limit of quantitation (LOQ), recovery, precision, accuracy and selectivity and a summary of validation parameters were presented in Table (1).

Table 1. Summary of validation parameters and linearity range of proposed procedure

Parameter	Method
Linear Range (µg/ml)	2 - 10
Slope	478.6X
Intercept	441.6
Regression co-efficient	0.998
Limit of Detection (µg/ml)	0.075
Limit of Quantification	0.23



(µg/ml)		

Linearity

Linearity of an analytical method is its ability to produce results that are directly, proportional to the concentration of analyte in samples. The range of the procedure is an expression off the lowest and highest levels of analyte that have been demonstrated to be determined with acceptable linearity, accuracy and precision. Agomelatine showed linearity in the concentration range of 2-10 µg/ml (r^2 = 0.998) for HPLC. Linearity was evaluated by determining five standard working solutions containing 2-10 µg/ml thrice in triplicate. Peak areas of agomelatine were plotted versus agomelatine concentration and linear regression analysis performed on the resultant curve. For HPLC method the linearity of calibration graphs and adherence of the system to Beer's law was validated by high value of correlation coefficient (0.998) and the standard deviation for intercept value was (10.97) as shown in Table (1).

Limit of detection and limit of quantification

Limit of detection is the lowest analyte concentration likely to be reliably distinguished from the limit of blank and at which detection is feasible, Whereas limit of quantitation is the lowest concentration at which the analyte can not only be reliably detected but at which some predefined goals for bias and imprecision are met[(15)]. The detection limit (LOD) and quantitation limit (LOQ) may be expressed as: [L.O.D. = 3.3*(SD/S) & L.O.Q. = 10*(SD/S)]. Where, SD = Standard deviation of the response and S = Slope of the calibration curve. The slope S may be estimated from the calibration curve of the analyte and the results were represented in Table (1) The LOD was found to be 0.075 µg/ml. The LOQ was found to be 0.23 µg/ml.

Precision

The precision of assay was determined with respect to both repeatability and reproducibility. An amount of the product powder equivalent to 100% of the label claim of agomelatine was accurately weighed and assayed. System repeatability was

determined by five replicate applications and three times measurement of a sample solution at the analytical concentration. The repeatability of sample application and measurement of peak area for active compound were expressed in terms of % RSD (relative standard deviation). Method repeatability was obtained from RSD value by repeating the assay three times in same day for intra-day precision. Inter-day precision was assessed by the assay of three sample sets on different days (inter-day precision). The intra-day and inter-day variation for determination of agomelatine was carried out at three different concentration levels 4, 6, 8 µg/ml as shown in Table (2) and Table (3).

Table 2. Method precision (intra-day and inter-day) (n = 3)

Concentration	**Intra-day µg/ml**	**% RSD** (Acceptance criteria ≤ 2.0%)	**Inter-day µg/ml**	**% RSD** (Acceptance criteria ≤ 2.0%)
4	3.96	0.51	3.93	1.21
6	5.91	1.34	5.94	1.08
8	7.85	1.41	7.89	1.58

Table 3. Recovery study data of agomelatine

Std agomelatine µg/ml	**Sample agomelatine µg/ml**	**Total Conc.**	**Total amount from standard graph µg/ml**	**Recovery of standard**	**% Recovery of standard**
2	2	4	3.9	1.91	95
4	2	6	5.9	3.9	97.5
6	2	8	8	6	104.6

Accuracy

The accuracy of assay was determined by interpolation of replicates (n=3) peak areas of three accuracy standards (2, 4, 6 µg/ml) from a calibration curve prepared as previously described. In each case the accuracy was calculated. The mean resultant concentrations, for three accuracy standard, were 101±0.37, 98.78±0.46 and 98.94±0.29 (mean ±standard deviation), respectively. Table (4)

Table 4. Accuracy

Level	**Amount of drug added (µg/ml)**	**% Recovery**	**Mean ± SD**	**%RSD (n=3)** (Acceptance criteria ≤ 2.0%)
S1: 50%	2.02	101	101±0.37	0.182
S2: 50%	2.01			
S3: 50%	2.03			
S1: 100%	3.94	98.78	98.78±0.46	0.31
S2: 100%	3.97			
S3: 100%	3.93			
S1: 150%	5.96	98.94	98.94±0.29	0.282
S2: 150%	5.95			
S3: 150%	5.92			

Robustness

To evaluate HPLC method robustness a few parameters were deliberately varied. The parameters included variation of C_{18} columns from different manufacturers, flow rate, percentage of acetonitrile in the mobile phase. Each parameters (except columns from different manufacturers and solvents of different lots) was changed at three levels, (for

flow rate at 0.8, 1 and 1.2 ml/min. and for mobile phase 60:40, 65:35 and 70:30), and examined. One factor at the time was changed to estimate the effect. Thus, replicate injections (n=3) of standard solution at three concentration levels were performed under small changes of two chromatographic parameters. Results indicate that the selected factors remained unaffected by small variations of these parameters. and no significant influence on the determination. Also, insignificant differences in peak areas and less variability in retention time were observed. Table (5)

Table 5. Robustness

Parameter	Change	Peak area		%RSD
Flow rate (ml/min.)	0.8	3956.23	Mean **3921.26**	0.750
		3942.12		
		3865.45		
	1 (Normal)	**3745.86**		1.022
	1.2	3658.55	**3659.07**	0.412
		3644.30		
		3674.23		
Mobile phase	60:40	3843.52	**3826.92**	0.486
		3822.11		
		3815.13		
	65:35 (Normal)	**3762.14**		0.987
	70:30	3663.22	**3655.02**	0.562
		3654.3		
		3647.54		

Stress study

The results of stress testing studies indicated a high degree of selectivity of this method for agomelatine. Typical chromatograms obtained from the assay of pure sample and stressed samples are shown in Figures (2,3). The peaks obtained were sharp and have clear baseline separation. Also, all the main degradation products were separated from the parent compound. Agomelatine was found to be stable under dry heat conditions and also no decomposition was seen on exposure of solid drug powder to light, which was kept for five days. The drug was unstable under basic stress conditions when kept for 15 min under room temperature. The drug was degraded approximately to 86%. Also it was unstable in acidic conditions when kept for 15 min at room temperature. The drug was degraded approximately to 91%. When kept under oxidative stress conditions with 1% H_2O_2 for 30 min at room temperature, the drug was degraded to around 68%. In neutral conditions when the drug was refluxed with water at 80°C for 6 h, around 75% degradation was shown. The stability of stock solution was determined by quantitation of agomelatine and comparison to freshly prepared standard. No significant change was observed in the stock solution response, relatively to freshly prepared standard http://www.ijpsonline.com/viewimage.asp?img=IndianJPharmSci_2009_71_1_24_51946_t10.jpg.

Application

The proposed method was applied to the determination of agomelatine in Aomela tablets. The result of these assay yielded 99.68%±0.63 (%RSD=0.86) of label claim of the tablets. The results of the assay indicate that the method is selective for the assay of agomelatine without interference from the excipients used in these tablets Table 6 .

Table 6. Analysis of commercial formulation

Tablet	Dosage	Sample concentration	Sample estimated	SD[1]	% RSD[2] n=3
Agomela	25 mg	6 µg/ml	99.68	±0.63	0.86

[1]standard deviation [2]Relative standard deviation

Conclusion

Proposed isocratic RP-HPLC method can be used as a stability indicating assay as well as for the assay of agomelatine in tablet dosage form. The advantages of the proposed method is the first method as simultaneous determination of agomelatine in the presence of its degradation product as a quality control method. So, the proposed method is simple, economy, accurate, precise, reliable and suitable for the routine quality control and stability indicating studies of agomelatine for raw material and pharmaceutical preparations.

Acknowledgements

Authors express sincere thanks to “Division of quality control of anticancer Drugs.”, Dr.Ali Mosa, in NODCAR , Egypt, for providing facilities to complete this work.

References

1- Venkat Rao P., Prabhakar T., Naveen R., Ramakrishna S. and Trinath G.; Research Journal of Pharmaceutical, Biological and Chemical Sciences, Volume 1 Issue 2 Page No. 446 (2010).

2- De Bodinat C, Guardiola-Lemaitre B, Mocae¨ r E, et al.: Nat Rev Drug Discovery, volume 9 page 628e642 (2010).

3- Grassi-Zucconi G, Semprevivo M, Mocaer E, et al.: Brain Resereach Bull, volume 39 page 63e68 (1996).

4- Weibel L, Turek F, Mocaer E, et al. Brain Resereach Bull, volume 8 page 207e211 (2000).

5- Dolder CR, Nelson M, Snider M.: Ann Pharmacother, volume 42 page 1822e1831 (2008).

6- Demyttenaere K. : Euro Neuropsychopharmacol volume 21:S703eS709 (2011).

7- Singh S. and Bakshi M.; Pharmaceutical Technology on-line 24: 1-14 (2000).

8- Klick S., Muijselaar V., Waterval J., Eichinger T., Korn C., Gerding T.; Pharmaceutical Technology, February: 48-66(2005).

9- Baerstichi S.: 1st edition, Chapter 6 Informa Healthcare, London, U.K., pp. 459–469 (2005).

10- Patil SR, Nerurkar KK, Kalamkar AM, Pukale V, Mangaonkar KV, Pingale SG : J Mass Spectrom 47:23–28 (2012)

11- Tadashi O M , Hideki Hattori • KT, Osamu SH, : J Forensic Toxicol in short communication (2014).

12- United States Pharmacopeia 28, National Formulary 23.: Rockville in (2005) pp. 2748–2751 (General Chapter 1225) (2005).

13- Proceedings of the International Conference on Harmonisation of Technical Requirements of Registration of Pharmaceuticals for Human Use. Validation of Analytical Procedures ICH-Q2A, Geneva (1995).

14- Proceedings of the International Conference on Harmonisation of Technical Requirements of Registration of Pharmaceuticals for Human Use (ICH Harmonised Tripartite Guidelines). Validation of Analytical Procedures: Methodology ICH-Q2B, Geneva (1996).

15- Clinical and Laboratory Standards Institute. Protocols for Determination of Limits of Detection and Limits of Quantitation, Approved Guideline. CLSI document EP17. Wayne, PA USA: CLSI; (2004).