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QbD Approach for Analytical Method Development and Validation of Serotonin by Spectroscopic Method

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ABSTRACT

According to International Conference on Harmonization (ICH Q8 [R2]) guidelines, an experimental work was planned for both spectroscopic method development and its validation. QbD (Quality-by-Design) approach was implemented for spectroscopic method development and its validation. The research work demonstrated that the UV is valid for the determination of assay of Serotonin. It describes the materials and methods used in experimental work. For performing experimental work analytical grade chemicals (methanol, water & ethanol) was used. The spectrophotometric method development and validated on UV spectrophotometer by using suitable solvent (ethanol, methanol & water) and detection was performed at 227nm.QbD approach was carried out for spectroscopic method development by varying 17 parameters and critical parameters were extracted by using principal component analysis and by observation. For all the variable parameters as stated in Ishikawa diagram, the absorbance was recorded over the concentration range.

INTRODUCTION

Analytical methods play an important role supporting implementation of QbD in process pharmaceutical development and development and manufacturing. Analytical testing also plays prominent role in pharmaceutical development, risk assessment, process monitoring and control and continuous quality assessment throughout the product. QbD is well-established in development and manufacture of pharmaceutical drug substance and drug product and is discussed in ICH Q8, [1] Q9 and Q2. The same QbD approach can be applied to analytical procedures as per ICH Q2. In addition, there is now a technique to definitively link the data to its intended use. These are exciting times for testing laboratories and the users of the data they produce. The knowledge obtained during development helps to justify the establishment of a design space, process control strategy and set point within the (regulatory approved) design space. Materials made within the design space will produce an acceptable product, and changes within the design space are regulatory acceptable. QbD approach suggests looking into the quality of analytical process during the development stage itself. It says that quality should be built into the process design rather than testing into final results of analytical process. QbD is defined as a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding based on sound science and quality risk management‖. In alignment with the approach proposed in the draft FDA (Food and Drug Administration) guidance for process validation, a three-stage approach can be applied to method validation.[2]

Serotonin or 5-hydroxytryptamine is a monoamine neurotransmitter. Biochemically derived from tryptophan, serotonin is primarily found in the gastrointestinal tract, blood platelets, and the central nervous system of animals, including humans. Neurotransmitters are chemicals that allow signal transmission, and thus communication among the nerve cells (neurons). One of the neurotransmitters used by neurons throughout the brain is 5-hydroxytryptamine also known as serotonin (5-HT). Serotonin is produced in and released from neurons that originate within discrete regions in the brain. Serotonin was originally discovered by Italian Vittorio Erspamer in Rome in [1] and American scientists, Maurice M. Rapport, Arda Green, and Irvine Page of the Cleveland Clinic isolated and named in 1948 [2]. The name "serotonin" is often referred to as a misnomer and reflects the circumstances of the compound's discovery [3]. It was initially identified as a vasoconstrictor substance in blood serum hence "serotonin", a serum agent affecting vascular tone. This agent was later chemically identified as 5-

hydroxytryptamine [3] and, as the broad range of physiological roles were elucidated, 5-HT became the most widely used and preferred name in the pharmacological field. Serotonin is a central and a peripheral neurotransmitter. It is biochemically synthesized from the amino acid tryptophan and it plays a great role in regulating various physiological functions such as sleep, hemostasis, and behavior regulation; in pathological conditions such as carcinoid syndrome, hypertension, thrombosis, and in cardiovascular diseases as well as psychiatric and neurological disorders such as schizophrenia, Huntington's disease, including many others [4,5]. Serotonin is widespread in nature and can be found in foods, nuts, and animals.

Stage1. Method Design: Define method requirements and conditions and identify critical controls.

Stage2. Method Qualification: Confirm that the method is capable of meeting its design intent. Stage3.Continued Method Verification: Gain ongoing assurance to ensure that the method remains in a state of control during routine use. A critical function of Stage 1 is the design of an Analytical Target Profile (ATP) for the method. To design the ATP, it is necessary to determine the characteristics that will be indicators of method performance for its intended use. These are selected from the performance characteristics described in ICH Q2 as per the traditional approach. Instead of being applied in a tick box manner, they are

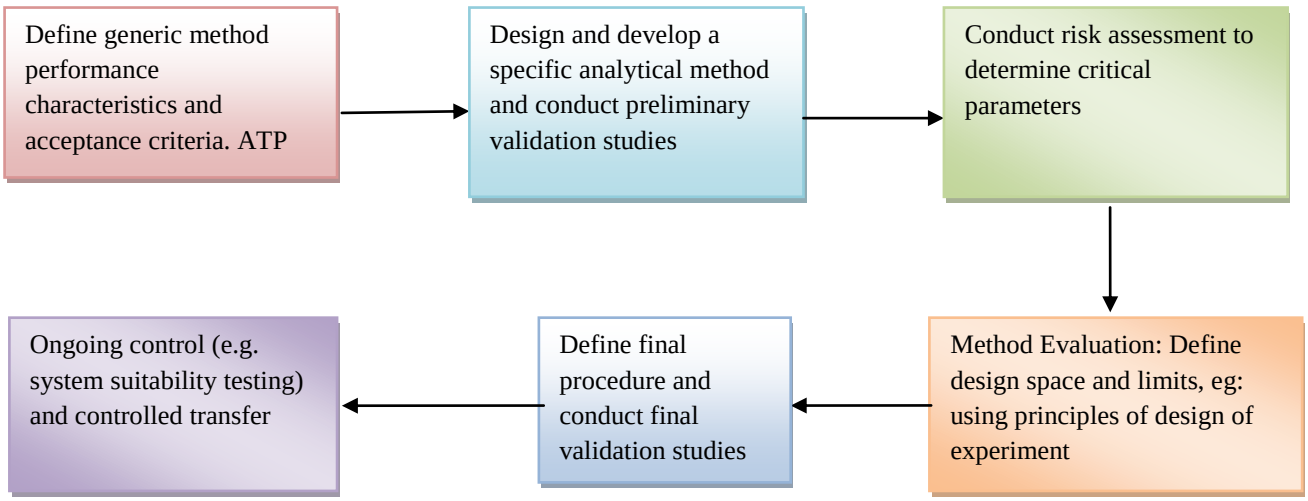

Fig 1-QbD work flow

Investigated by a risk assessment exercise as described in ICH Q9 in combination with carefully designed development studies to identify the critical method and sources of variation. Variables are then investigated by robustness and ruggedness experiments to understand the functional relationship between method input variables and each of the

method performance characteristics and the results are compared to the desired outcome defined in the ATP. From this, one can identify a set of operational method controls. Also, having evaluated the critical method parameters and gained a better understanding of the method through structured experimentation.[3] Addition to validating the method characteristics as per regulatory guidance, verifying the accuracy and precision provides additional understanding of the method‘s measurement uncertainty and confirms conformance to the previously defined method performance requirements (ATP). This can be accomplished through a joint accuracy and precision. Serotonin (5-hydroxytryptamine, 5-HT) serves as a central and peripheral neurotransmitter/neuromodulator and has growth factor-like action in the developing nervous system [1, 2]. Serotonin mediates a range of critical behaviors [3], and it has been linked to a number of neuropsychiatric disorders including anxiety, depression, obsessive-compulsive disorder (OCD), and autism [4-6]. Serotonin and related compounds, including its precursor amino acid tryptophan (TRP) and major metabolite 5-hydroxy- tryptophan[13]. So attempt has made to develop fast, simple, economical and precise method for estimation of serotonin by applying QbD approach by Spectroscopic Method

MATERIALS AND METHODS

Standards and materials Standard materials were obtained from the following suppliers: N-methyl serotonin (NMS) oxalate salt and perchloric acid were from Aldrich Chemicals (Milwaukee, WI); serotonin creatinine sulfate, l-tryptophan (TRP), indole-3-propionic acid (IPA), 5-hydroxy-indole-3-acetic acid (5-HIAA), ascorbic acid, and sodium metabisulfite were from Sigma Chemicals (St. Louis, MO); and indole-3-acetic acid (IAA) was from Across Chemicals (Pittsburgh, PA). Stock solutions of all standards were prepared by weighing the appropriate amount of the standard material to obtain 10.0 mg of the free base (or acid). The free base material was dissolved in 100 mL of 0.2% ascorbic acid, resulting in stock solutions of 0.1 mg/mL. The stock solutions were further diluted in 0.2% ascorbic acid to the 440 Reproduction (photocopying) of editorial content of this journal is prohibited without publisher's permission. Journal of Analytical Toxicology, Vol. 27, October 2003 appropriate concentrations for the working standards. All standard were stored at -80~ In patients with sleep disorders and altered circadian rhythms, such as occur in jet lag, night shift work, and various neuropsychiatric disorders, oral administration of melatonin can provide the necessary resynchronization of those cycles, at dosages ranging from 0.3 to 8 mg. Synthesis of melatonin from the amino acid tryptophan is decreased by exposure to magnetic

fields and by the aging process. Melatonin is a potent scavenger of free radicals and exerts direct inhibition of cancer growth. Various cancer types have been shown to be responsive to oral melatonin (10-50 mg daily), including breast cancer, non-small-cell lung cancer, metastatic renal cell carcinoma, hepatocellular carcinoma, and brain metastases [5] from solid tumors. Serotonin has also been reported to lower LDL (Low Density Cholesterol) and total cholesterol levels. Abnormally low melatonin levels have been theorized to be a factor in multiple sclerosis, coronary heart disease, epilepsy, and postmenopausal osteoporosis. These reports, while preliminary, serve to further illustrate the wide range of potential effects exerted by melatonin.[6,7'8] It is soluble in water; soluble in methanol, Ethanol, slightly soluble in alcohol and in chloroform; and very slightly soluble in acetone.

The present work aims at systematic development of a simple, rapid and highly sensitive method for the analysis of Serotonin by QbD approach.

All chemicals used during the project work were either AR. The various reagents and chemicals used during experimental work are purchased from different sources and in details given in Table number 1.

Table 1: Chemicals and instruments

Sr. No.	Name of Chemical	Source	Sr. No.	Name of Equipment	Source
1.	Water	D/W	1.	UV	Shimadzu, Model: UV-1800
2.	Methanol	Analytical grade	2.	Electronic weighing balance	Shimadzu BL- 220 H
.	Ethanol	Analytical grade	3.	Sonicator	The ultrasonic's PCi Analytics Sonicator

Methods Preliminary solubility study of drug:

Solubility of the drug was determined at 28±1^{0}C. A small quantity of standard drug was dissolved in different solvents like distilled water, ethanol, methanol, acetonitrile, alcohol, chloroform, acetone. The Qbd approach was applied by considering various parameters of solubility and its absorbance. Details about it is mentioned in Table 2-9

Preparation of Stock solution: Preparation of standard stock solution of Serotonin:

10 mg of Serotonin accurately weighted by electronic balance and dissolved in 80ml of double distilled water in 250ml conical flask. Content of flask was kept for stirring on magnetic stirrer for 10 min and transferred to 100ml volumetric flask. Conical flask was rinsed by 20ml of double distilled water and this water was used to make up volume 100ml of volumetric flask to give conc. of 100µg/ml.

Preparation of working standard solution of Serotonin:

The working solution of Serotonin was prepared by further diluting the stock solution. Then pipette out 0.2ml, 0.4ml, 0.6ml, 0.8ml, 1.0ml & 1.2ml of solution and make up to 10ml leads to 2µg/ml, 4µg/ml, 6µg/ml, 8µg/ml, 10µg/ml & 12 µg/ml concentration solution. This solution was estimated by UV spectrophotometer by using Methanol as blank at 227nm.

Fixing of wave length

After selecting the suitable solvent, the fixing of the λmax for the proposed method is very important. This can be done by scanning the drug sample (Serotonin) solution in Methanol in the range of 400nm-200nm and the most repeated maximum absorbance with linearity and repeatability can be fixed as λmax for the drug. And in the proposed method for Serotonin drug shows maximum 227 nm. With more linearity, repeatability (ruggedness) and the λmax was fixed as 227nm.

Linearity and range:

For linearity study from the working standard at different concentration 2, 4, 6, 8, 10 and 12 µg/ml of drug solution were placed in 6 different 10ml volumetric flask volume was made up to the mark with Methanol. Absorbance was measured at 225nm. The obtained data of absorbance of standard stock solution presented in table no 10 calibration plot represented by Figure 4.

Accuracy and recovery study:

This study was carried out using the stock solution (100µg/ml). Take three concentrations 8 µg/ml, 10µg/ml, and 12µg/ml. And take six reading of these concentrations. Calculate the % Relative Standard Deviation (RSD) of the concentration. Results within the range of ensuring

an accurate method as well as indicate non-interference with the excipients of formulation. The accuracy of the proposed methods was assessed by recovery studies at three different levels i.e. 80%, 100%, 120%. The recovery studies were carried out by adding known amount of Serotonin solution of the drug to pre-analyzed tablet solutions. The resulting solutions were then reanalyzed by proposed methods.

Intra-day precision (repeatability) and inter-day precision study (intermediate precision): The standard stock solution of Serotonin was Prepared. Prepare the three concentration of (8, 10, and 12 µg/ml), by using mobile phase methanol. Take λmax at the intraday and inter day. Calculate the % RSD. Variation of results within the day (intra-day), Variation of result between days (inter day) were analyzed. Intraday precision was analyzing Serotonin for three times in the same day at 227nm. Inter-day precision was determined by analyzing the drug different day for three days at 227nm. Precision data for Serotonin at 225nm

Reproducibility:

Reproducibility is assessed by mean of an inter laboratory trial. The absorbance readings were measured at 227nm at different laboratory using another spectrophotometer and the value obtained were evaluated using t-test to verify their reproducibility data for Serotonin at 227nm is recorded.

Limit of Detection & Limit of Quantitation:

The limit of detection and quantification of drug are calculated with the standard deviation and slop.

$$LOD = \frac{3.3\sigma}{S} \quad LOQ = \frac{10\sigma}{S}$$

Where,

σ = standard deviation

S = slope of calibration curve

Stability of Sample:

Samples prepared for repeatability study were preserved for 24hours at room temperature 28°C and analyzed on the following day to test for short-term stability. The sample of 4µg/ml drug solution was prepared by suitable dilution with diluents and absorbance were taken at 225nm against the blank. The stability of sample was found to be more than 10 hrs.

Acid degradation:

The preparation of 0.01N hydrochloric acid (HCl) was done by diluting 0.085 ml of conc. HCI to 100 ml of distilled water. Serotonin was accurately weighted and was transferred to a labeled round bottomed flask. Reflux the sample for 2 hrs. And pipette out 1ml to 10 ml volumetric flask and adjust with mobile phase. Volume of 20µl was injected into the system for chromatographic analysis and results of all chromatograms were compared to see whether degradation occurred or not. The degradation of drug is not more than 30%.

Base degradation: The 0.01N Sodium Hydroxide (Noah) was prepared by dissolving 0.04 gm of sodium hydroxide pellets in 100 ml of distilled water. The solution was standardized with 0.01 N HCl as per Indian Pharmacopoeia (I.P). Serotonin was accurately weighted and was transferred to a labeled round bottomed flask. Reflux the sample for 2 hrs. And pipette out 1ml to 10 ml volumetric flask and adjust with mobile phase. Volume of 20µl was injected into the system for chromatographic analysis and results of all chromatograms were compared to see whether degradation occurred or not. The degradation of drug sample is not more than 22%.

Neutral condition:

Weight accurately 10 mg drug and transferred in to100 ml water in round bottom flask. Reflux it for 2 hours. Pipette out 1ml into 10 ml volumetric flask and adjust with mobile phase.

Photo stability study:

Photo stability was performed by placing 10 mg of Serotonin in daylight for 24 hours. The samples were diluted with methanol up to 100ml in a volumetric flask. Pipette out 1 ml

sample diluted up to 10 ml by mobile phase. Volume of 20µl was injected into the system for chromatographic analysis.

Dry heat: Standard Serotonin was placed in an oven at 60°C for 2 hours to study dry heat degradation. 10 mg drug samples were diluted with methanol up to 100ml in a volumetric flask. Pipette out 1 ml and were diluted up to 10 ml by mobile phase. Volume of 20µl was injected into the system for chromatographic analysis and results of both chromatograms were compared to see whether degradation occurred or not.

Assay Procedure -

Take weight of 10 tablets of any brand of Serotonin tablet. Crush the tablet in the motor pestle. Accurately weigh the quantity of powder equivalent to 10mg of drug in 100ml volumetric flask and add ethanol to adjust the volume up to 100ml. Pipette out the 1ml into 10 ml volumetric flask make the volume with mobile phase to get conc. 10µg/ml and analyze the reading on HPLC. Calculate the percentage purity of tablet.

RESULT AND DISCUSSION

Preliminary solubility study of drug:

Solubility of the drug was determined at 28°C. A small quantity of standard drug was dissolved in different solvents. Implementation of QbD approach for the Spectrophotometric method development as per ICH Q8(R2) guidelines for estimation of Serotonin by varying various parameters and these variable parameters were designed as per Ishikawa. Critical parameters for the development of zero order Spectrophotometric method are considered as various solvent, sample preparation of tablet, wavelength at 220 & 222 nm, slit width as 1, scan speed and sampling interval (0.05, 0.1, 0.2, 0.5, 1.0, and 2.0)

Determination of Variable Parameters

According to QbD approach, the first step is to determine the variable parameters for the respective method. Thus, the variable parameters for both the spectrophotometric methods were designed as Ishikawa diagram (Figures 2). For all the variable parameters as stated in Ishikawa diagram, the absorbances were recorded over the concentration range according to respective method. Working solution was scanned from 400 to 200nm and three peaks were observed at wavelengths225nm, 227nm. These three wavelengths were used as variable

parameters. Also, the solubility was studied in various solvents including distilled water, and methanol. The sharpness of spectra was compared for selection of critical parameter. Scan speed was varied as fast, medium, slow, and very slow over the range 400– 200nm, while slit width and sampling interval were varied in particular ranges of 0.1, 0.2, 0.5, 1.0, 2.0, and 5.0nm and 0.05,0.1,0.2,0.5,1.0 and 2.0nm, respectively. For the estimation of melatonin, two types of sample preparations were selected and evaluated. Tablets were formulated as per the master formula and were used in method development. Average weight of tablets was noted and tablets were triturated. Tablet powder equivalent to average weight was taken for study and evaluated for the method development. Recovery study was carried out at three levels 80%, 100%, and 120%.

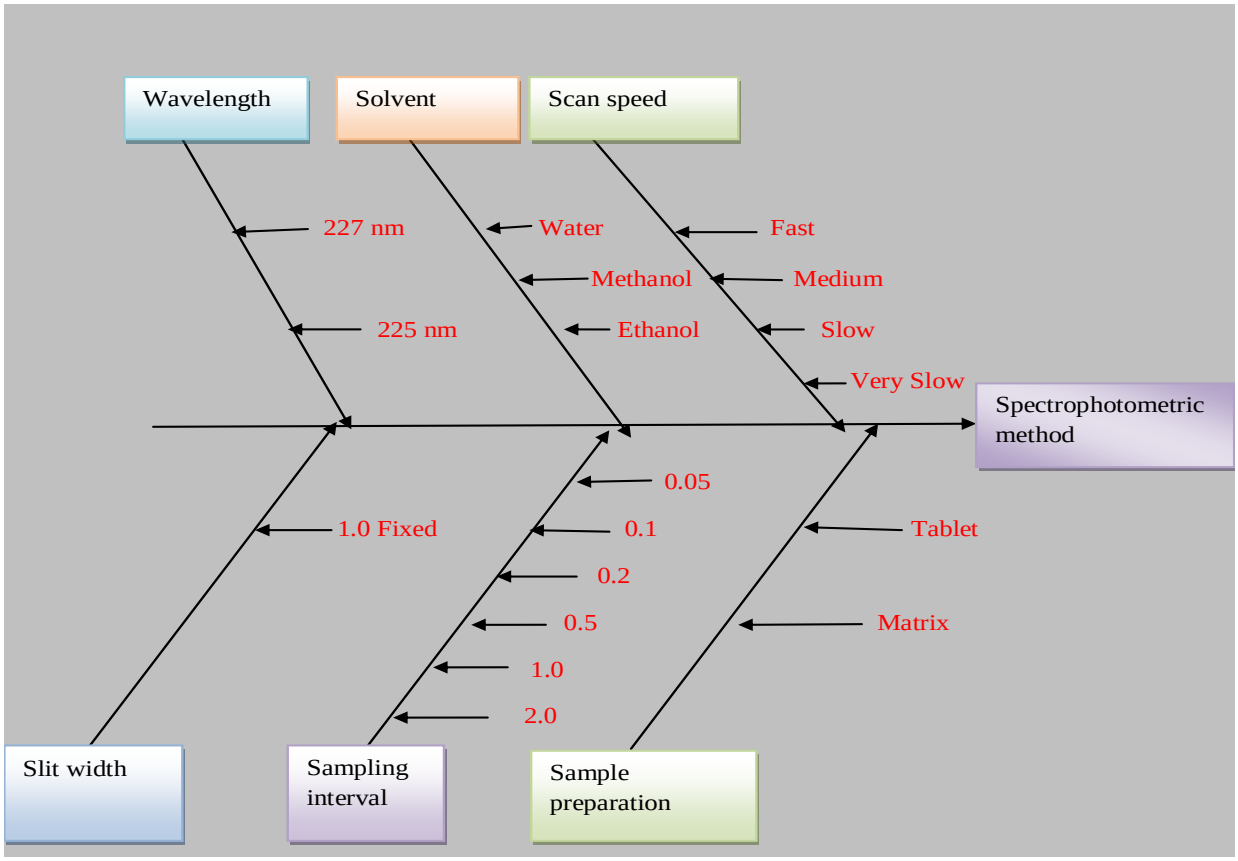

Figure 2: Ishikawa diagram.

Table 2: Solubility study in different Solvent by UV-absorbance

Solvent	Concentration µg /ml	Absorbance	Solvent	Concentration µg /ml	Absorbance
Water	2	0.047	**Methanol**	2	0.205
	4	0.072		4	0.334
	6	0.095		6	0.597
	8	0.102		8	0.667
	10	0.188		10	0.764
	12	0.215		12	0.896
Ethanol	2	0.0047	**Methanol (With stirring)**	2	0.313
	4	0.010		4	0.533
	6	0.013		6	0.739
	8	0.064		8	0.970
	10	0.092		10	1.248
	12	0.105		12	1.474

Table 3: Effect of Stirring on absorbance at (ʎmax-227nm) As per Stirring time

Solvent	Conc. µg/ ml	Effect of Stirring on absorbance at (ʎmax-227) As per Stirring time							
		0 min		2 min		4 min		6 min	
		225nm	227nm	225nm	227nm	225nm	227nm	225nm	227nm
Methanol (With Stirring)	2	0.311	0.313	0.327	0.329	0.339	0.341	0.354	0.357
	4	0.531	0.533	0.545	0.547	0.556	0.558	0.570	0.572
	6	0.737	0.739	0.0.751	0.753	0.765	0.767	0.779	0.781
	8	0.767	0.970	0.982	0.984	0.996	0.999	1.110	1.112
	10	1.246	1.248	1.260	1.262	1.274	1.276	1.880	1.882
	12	1.471	1.474	1.481	1.483	1.495	1.497	1.509	1.511
Solvent	**Conc. µg/ ml**	**Effect of Stirring on absorbance at (ʎmax-227) As per Stirring time**							
		8 min		**10 min**		**12 min**		**14 min**	
		225nm	227nm	225nm	227nm	225nm	227nm	225nm	227nm
Methanol (With Stirring)	2	0.365	0.368	0.373	0.375	0.386	0.388	0.394	0.397
	4	0.584	0.586	0.598	0.610	0.622	0.624	0.636	0.638
	6	0.793	0.795	0.807	0.809	0.821	0.823	0.835	0.837
	8	0.124	0.126	0.138	0.140	0.152	0.154	0.166	0.168
	10	0.194	0.196	0.208	0.210	0.222	0.224	0.236	0.238
	12	1.523	1.525	1.537	1.539	1.541	1.543	1.555	1.557

Table 4: UV Absorbance as per Instrumental Parameter (C.Q.A.) Con. 2ppm

Time interval		Fast Mode		Medium Mode		Slow Mode		Very Slow Mode	
		225 nm	227 nm	225 nm	227 nm	225 nm	227 nm	225 nm	227 nm
0.05	Abs.	0.311	0.312	0.310	0.311	0.310	0.311	0.309	0.310
	Time Req.	2.40 min	-	10.45 min	-	27 min	-	53.10 min	-
0.1	Abs.	0.311	0.312	0.310	0.311	0.309	0.310	0.309	0.310
	Time Req.	1.40 min	-	6 min	-	12 min	-	21 min	-
0.2	Abs.	0.312	0.313	0.310	0.311	0.309	0.310	0.309	0.310
	Time Req.	1 min	-	3 min	-	7 min	-	12 min	-
0.5	Abs.	0.311	0.312	0.310	0.311	0.309	0.310	0.309	0.310
	Time Req.	0.45 min	-	1.23 min	-	3.2 min	-	8 min	-
1.0	Abs.	0.312	0.313	0.310	0.311	0.309	0.310	0.309	0.310
	Time Req.	0.25 min	-	0.45 min	-	2 min	-	4.40 min	-
2.0	Abs.	0.312	0.313	0.309	0.310	0.309	0.310	0.309	0.310
	Time Req.	0.10 min	-	0.22 min	-	0.45 min	-	2.30 min	-

Table 5: UV Absorbance as per Instrumental Parameter (C.Q.A.) Con. 4ppm

Time interval		Fast Mode		Medium Mode		Slow Mode		Very Slow Mode	
		225 nm	227 nm	225 nm	227 nm	225 nm	227 nm	225 nm	227 nm
0.05	Abs.	0.531	0.532	0.530	0.531	0.530	0.531	0.529	0.530
	Time Req.	2.40 min	-	10.45 min	-	27 min	-	53.10 min	-
0.1	Abs.	0.531	0.532		0.531	0.529	0.530	0.529	0.530
	Time Req.	1.40 min	-	6 min	-	12 min	-	21 min	-
0.2	Abs.	0.532	0.533	0.530	0.531	0.529	0.530	0.529	0.530
	Time Req.	1 min	-	3 min	-	7 min	-	12 min	-
0.5	Abs.	0.531	0.532	0.530	0.531	0.529	0.530	0.529	0.530
	Time Req.	0.45 min		1.23 min		3.2 min		8 min	
1.0	Abs.	0.532	0.533	0.530	0.531	0.529	0.530	0.529	0.530
	Time Req.	0.25 min	-	0.45 min	-	2 min	-	4.40 min	-
2.0	Abs.	0.532	0.533	0.529	0.530	0.529	0.530	0.529	0.530
	Time Req.	0.10 min	-	0.22 min	-	0.45 min	-	2.30 min	-

Table 6: UV Absorbance as per Instrumental Parameter (C.Q.A.) Con. 6ppm

Time interval		Fast Mode		Medium Mode		Slow Mode		Very Slow Mode	
		225 nm	227 nm	225 nm	227 nm	225 nm	227 nm	225 Nm	227 nm
0.05	Abs.	0.738	0.739	0.736	0.737	0.740	0.741	0.742	0.743
	Time Req.	2.40 min	-	10.45 min	-	27 min	-	53.10 min	-
0.1	Abs.	0.738	0.739	0.736	0.738	0.740	0.741	0.742	0.743
	Time Req.	1.40 min	-	6 min	-	12 min	-	21 min	-
0.2	Abs.	0.737	0.738	0.738	0.739	0.740	0.741	0.742	0.743
	Time Req.	1 min	-	3 min	-	7 min	-	12 min	-
0.5	Abs.	0.738	0.739	0.739	0.740	0.740	0.741	0.742	0.743
	Time Req.	0.45 min	-	1.23 min	-	3.2 min	-	8 min	-
1.0	Abs.	0.738	0.739	0.740	0.741	0.741	0.742	0.742	0.743
	Time Req.	0.25 min	-	0.45 min	-	2 min	-	4.40 min	-
2.0	Abs.	0.738	0.739	0.740	0.741	0.742	0.743	0.742	0.743
	Time Req.	0.10 min	-	0.22 min	-	0.45 min	-	2.30 min	-

Table 7: UV Absorbance as per Instrumental Parameter (C.Q.A.) Con. 8ppm

Time interval		Fast Mode		Medium Mode		Slow Mode		Very Slow Mode	
		225nm	227 nm	225 nm	227 nm	225 nm	227 nm	225 nm	227nm
0.05	Abs.	0.969	0.970	0.973	0.974	0.977	0.978	0.980	0.981
	Time Req.	2.40 min	-	10.45 min	-	27 min	-	53.10 min	-
0.1	Abs.	0.969	0.970	0.973	0.974	0.977	0.978	0.980	0.981
	Time Req.	1.40 min	-	6 min	-	12 min	-	21 min	-
0.2	Abs.	0.969	0.970	0.973	0.974	0.977	0.978	0.980	0.981
	Time Req.	1 min	-	3 min	-	7 min	-	12 min	-
0.5	Abs.	0.969	0.970	0.973	0.973	0.977	0.978	0.980	0.981
	Time Req.	0.45 min	-	1.23 min	-	3.2 min	-	8 min	-
1.0	Abs.	0.969	0.970	0.973	0.974	0.977	0.978	0.980	0.981
	Time Req.	0.25 min	-	0.45 min	-	2 min	-	4.40 min	-
2.0	Abs.	0.970	0.970	0.973	0.974	0.977	0.978	0.980	0.981
	Time Req.	0.10 min	-	0.22 min	-	0.45 min	-	2.30 min	-

Table 8: UV Absorbance as per Instrumental Parameter (C.Q.A.) Con. 10ppm

Time interval		Fast Mode		Medium Mode		Slow Mode		Very Slow Mode	
		225 nm	227 nm	225 nm	227 nm	225 nm	227 nm	225 nm	227 nm
0.05	Abs.	1.247	1.248	1.251	1.252	1.255	1.256	1.258	1.259
	Time Req.	2.40 min	-	10.45 min	-	27 min	-	53.10 min	-
0.1	Abs.	1.247	1.248	1.250	1.252	1.254	1.257	1.258	1.259
	Time Req.	1.40 min	-	6 min	-	12 min	-	21 min	-
0.2	Abs.	1.247	1.248	1.251	1.252	1.255	1.256	1.258	1.259
	Time Req.	1 min	-	3 min	-	7 min	-	12 min	-
0.5	Abs.	1.253	1.248	1.251	1.252	1.255	1.256	1.258	1.259
	Time Req.	0.45 min	-	1.23 min	-	3.2 min	-	8 min	-
1.0	Abs.	1.247	1.248	1.251	1.252	1.255	1.256	1.258	1.259
	Time Req.	0.25 min	-	0.45 min	-	2 min	-	4.40 min	-
2.0	Abs.	1.247	1.248	1.251	1.251	1.255	1.256	1.257	1.259
	Time Req.	0.10 min	-	0.22 min	-	0.45 min	-	2.30 min	-

Table 9: UV Absorbance as per Instrumental Parameter (C.Q.A.) Con. 12ppm

Time interval		Fast Mode		Medium Mode		Slow Mode		Very Slow Mode	
		225 nm	227 nm	225 nm	227 nm	225 nm	227 nm	225 nm	227 nm
0.05	Abs.	1.473	1.474	1.477	1.478	1.480	1.481	1.484	1.485
	Time Req.	2.40 min	-	10.45 min	-	27 min	-	53.10 min	-
0.1	Abs.	1.473	1.474	1.477	1.478	1.480	1.481	1.484	1.485
	Time Req.	1.40 min	-	6 min	-	12 min.	-	21 min	-
0.2	Abs.	1.473	1.474	1.477	1.478	1.481	1.482	1.484	1.485
	Time Req.	1 min	-	3 min	-	7 min	-	12 min	-
0.5	Abs.	1.473	1.474	1.477	1.478	1.480	1.481	1.484	1.486
	Time Req.	0.45 min	-	1.23 min	-	3.2 min	-	8 min	-
1.0	Abs.	1.473	1.474	1.477	1.478	1.480	1.481	1.484	1.485
	Time Req.	0.25 min	-	0.45 min	-	2 min	-	4.40 min	-
2.0	Abs.	1.472	1.474	1.477	1.478	1.480	1.481	1.484	1.485
	Time Req.	0.10 min	-	0.22 min	-	0.45 min	-	2.30 min	-

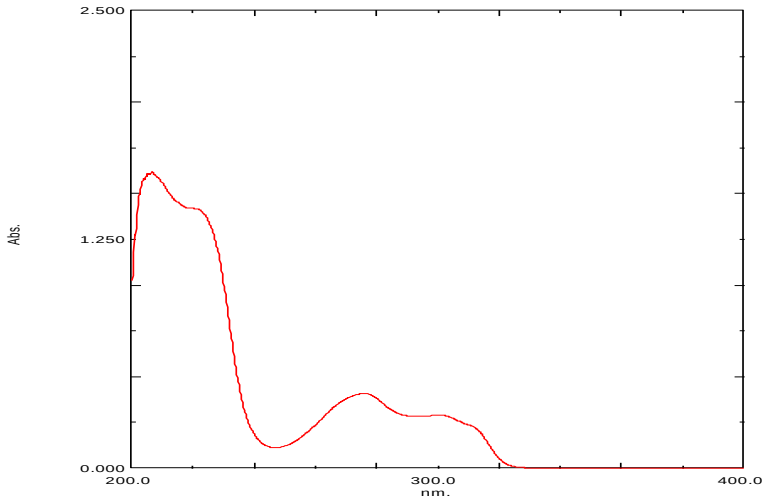

Figure 3: Fixing of wavelength for Serotonin

Linearity and range

The calibration curve obtained was evaluated by its correlation coefficient. The absorbance of the samples in the range of 2 to 12 mg/ml was linear with a correlation coefficient (R2) 0.997.

Table 10: Linearity and range for Serotonin at 227nm

Sr. No.	Concentration (µg/ml)	Absorbance
1.	2	0.313
2.	4	0.533
3.	6	0.739
4.	8	0.97
5.	10	1.248
6.	12	1.474

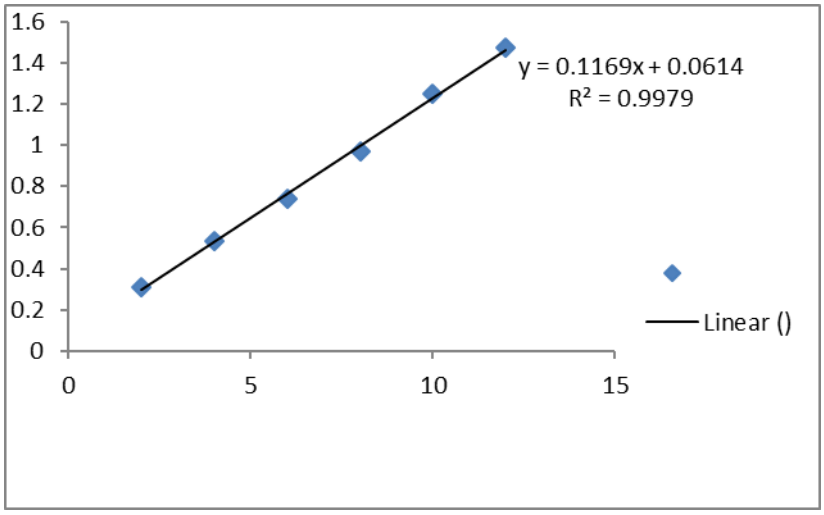

Figure 4: Linearity and range for Serotonin

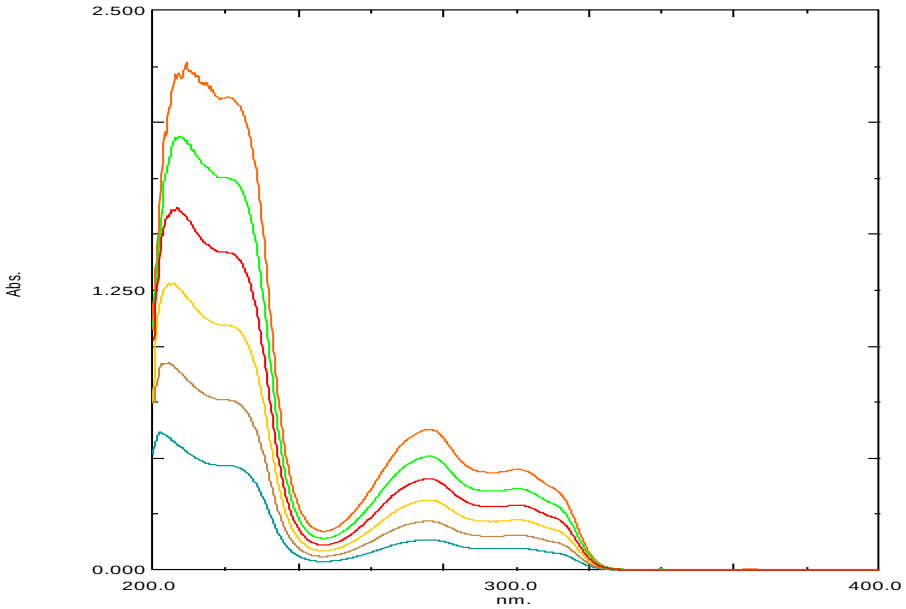

Figure 5: Accuracy sample overlay Serotonin

Table 11: Linearity Parameter

Parameter	Data
Range	2 µg/ml to 12 µg/ml
Correlation coefficient	0.997
Slope	0.116
Intercept	0.0614

Intra-day precision (repeatability) and inter-day precision study (intermediate precision):

Table 12: Precision data for Serotonin 227nm (Intra-Day)

Conc. (µg/ml)	Absorbance						AVG	SD	% RSD
8	0.413	0.413	0.414	0.415	0.416	0.417	0.414	0.0016	0.394
10	0.512	0.512	0.513	0.513	0.513	0.514	0.512	0.00075	0.147
12	0.637	0.637	0.639	0.639	0.639	0.638	0.638	0.00098	0.016

Table 13: Precision data for Serotonin at 227nm (Inter-Day)

Conc. (µg/ml)	Absorbance						AVG	SD	% RSD
8	0.37	0.385	0.385	0.386	0.386	0.387	0.384	0.0044	1.164
10	0.46	0.470	0.470	0.471	0.471	0.471	0.470	0.0081	0.172
12	0.59	0.594	0.595	0.596	0.596	0.596	0.595	0.0009	0.0163

Limit of Detection & Limit of Quantization:

$$LOD = \frac{3.3\sigma}{S} \quad LOQ = \frac{10\sigma}{S}$$

Where,

σ = standard deviation

S = slope of calibration curve

Table 14: Limit of Detection & Limit of Quantization

LOD	LOQ
12.43	37.67

Stability of Sample:

Table 15: Stability of Sample

Sr. No.	Conc. (µg/ml)	Time (min)	Absorbance at 222nm
1.	4	0	0.525
2.	4	30	0.525
3.	4	60	0.525
4.	4	90	0.526
5.	4	120	0.525
6.	4	150	0.526
7.	4	180	0.526
8.	4	210	0.527
9.	4	240	0.526
10.	4	360	0.524
11.	4	480	0.525
12.	4	600	0.523

Table 16: Degradation Studies

Stress condition	% Degradation Observed	Remarks
0.01N NaOH	91	Unstable
0.01N HCl	8	Stable
Oven	8	Stable
Water	22	Unstable
Oxidation	75	Unstable

Implementation of QbD approach was carried out by studying variable parameters in the analytical method development. Critical parameters were extracted by observation of results as well as performing principal component analysis. Also, each method was validated according to ICH Q2 (R1) guidelines. Degradation Studies factors are mentioned in Table 16.

Table 17: Critical parameters extracted for method

Critical parameters extracted		By Principle Component Analysis	
By observation Parameter	Extracted result	Parameter	Extracted Result
Solvent	Methanol	Wavelength	227 nm
		Scan speed	Fast
Sample preparation	Tablet	Slit width	1
		Sampling interval	2

Table 18: Statistical data of Validation

Parameters	Method
λ max	227nm
Linearity range	2 µg/ml to 12 µg/ml
Regression equation	y = 0.116x + 0.061
Correlation coefficient	0.997
LOD	12.43 µg/ml
LOQ	37.67 µg/ml
Precision (% R.S.D.) Intra-day	0.016 -0.394
Inter-day	0.0163 - 1.164

A zero order spectrophotometric method has been developed and validated for the determination of Melatonin in pharmaceutical formulation. QbD approach was carried out by varying 19 parameters and critical parameters were extracted by using principal component analysis and by observation. The extracted critical parameters are summarized in Table 17.

Melatonin followed linearity in the concentration range of 2– 12 µg/ml. The proposed method was applied for pharmaceutical formulation and percentage label claim was found to be 99.05%. The amount of drug estimated by proposed method was in good agreement with the label claim. The % recovery for tryptophan was found to be 100.1%. The method was found to be precise as indicated by the inter-day and intra-day analysis showing % RSD less than 2. There was no any interference of excipients showing that the method was specific. Limit of detection and limit of quantitation were 12.43 and 37.67 µg/ml, respectively. The result did not show any statistical difference between different solvents and different wavelengths suggesting that the method developed was robust. The statistical data of validation is summarized in Table 18.

CONCLUSION

The proposed methods were found to be accurate, precise, and economical and can be useful for routine quality control analysis of Serotonin in pharmaceutical dosage form. Implementation of QbD approach resulted in more robust methods which can produce consistent, reliable, and quality data throughout the process and also save time and money.

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