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A Demonstration of an Analytical Method Development and Validation Process Using the Identification and Assay of Ondansetron hydrochloride dihydrate in Ondansetron hydrochloride 4mg/5ml Syrup (Nausetron) by Reversed-Phase High Performance Liquid Chromatography

ABSTRACT: *The main purpose of this paper is to attempt to give a detailed step by step explanation of how data is collected and statistically treated during an analytical method development and validation protocol for Quality Control of Pharmaceutics. In most cases, modification to a given manufacturing process, formulation or synthetic pathway during a drug development program may necessitate changes to existing analytic methods. As a consequence, this change may require additional transfer and or validation studies. Effective method development in a pharmaceutical industry is aimed at ensuring that analytical methods meet the objectives required at each stage of drug development using the least resources available of which developmental time and cost are the main targets. Method validation involves demonstrating that these methods are suitable for their intended use as required by regulatory agencies. Method transfer is the formal process of assessing the suitability of methods in another laboratory. These studies involve numerous steps taken, according to a given Standard Operating Procedure (SOP) to collect data. Data analysis usually encompasses confusing mathematical manipulations that may require extensive knowledge of statistics. Although most papers and articles on method development and validation indicate how data is collected, very few give the final acceptance result and show how it was calculated. This paper therefore fills this gap by illustrating, following a hypothetical Standard Operating Procedure: MUJ-256-B dated 16th July 2015 from a hypothetical JBXY Pharmaceuticals, how to develop and validate a method to identify and quantitatively determine the concentration of the active pharmaceutical ingredient; (±) 1, 2, 3, 9-tetrahydro-9-methyl-3-[(2-methyl-1H-imidazol-1-yl) methyl]-4H-carbazol-4 one, monohydrochloride, dihydrate (Ondansetron hydrochloride dihydrate) in Ondansetron hydrochloride 4mg/5ml Syrup (Nausetron) by Reversed-Phase High Performance Liquid Chromatography.*

Keywords: *Acceptance Criteria, Hypothesis Testing, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use; Method Transfer; Ondansetron; Pharmaceutical; Statistics; Reversed-Phase; Biostatistics; Statistical Treatment; Quality Assurance; Quality Control.*

INTRODUCTION

A method is a set of experimental conditions designed to create a good analysis of a particular sample. Method development encompasses many stages and may take a long time to complete depending on the complexity and goals of the method (Waters Corporation, 2011). A typical outline of a method development and validation protocol in any pharmaceutical industry will include the following steps:

Understanding the Sample

It is very important to know the sample's composition and properties; particularly the properties that are most likely to be affected by the analytical method. For example, questions should be asked about the sample matrix or excipients to determine what compounds other than the analyte are present in the sample, about the concentration to determine how much of the compound is present and within what the concentration range, about the quantity to find out how many compounds are present in the sample and finally about both chemical and physical properties such as p^{Ka} values, molecular size, molecular weight, electrical charge, sample solubility, sample volatility, sample stability, sample toxicity, sample hydrophobicity (water affinity) polarity, chemical reactivity, biological reactivity and UV-visible spectra (Waters Corporation, 2011).

Defining Method Goals

This step is often overlooked but is critical to the success of the method because it answers the question on why the sample is being analysed. The goal may be: detection so as to find out if the compound is present, quantitation so as to see how much of the compound is present, identification to know what the compound is, characterisation to identify the compound's properties and purification or isolation to collect the compound for further use (Waters Corporation, 2011).

Determining Analysis Requirements

These are variables mainly associated with method goals. They attempt to answer the question on what needs to be done or known to attain the goals. If the goal is detection for example, what detection technique can be used to analyse the sample? is the sample UV absorbing? can the sample be ionised? does it have observable thermal characteristics and the like? If the goal is quantitation, how will it be quantified (e.g., using internal standard, external standard, absolute detection)? what is the concentration range or sample amount? how many samples are needed? what levels of accuracy and precision are required? If the goal is identification; how will the compound be identified (e.g., what detection technique will be used)? how will the sample purity be ensured? (e.g., UV spectral purity,

percent area)? If the goal is characterisation; what properties or property levels are needed to determine? If the method goal is purification or and isolation; is there need to isolate purified material? is there need to recover 100% of the sample? If it's about the sample matrix; is there more than one matrix before analysis? will the sample matrix interfere with the analysis? If it's about sample properties; does the analysis technique allow the determination of sample properties? (Waters Corporation, 2011).

Conducting Research

This is important so as to determine if the analysis has been performed before. Previously developed methods with quantitation and sample matrices that are close to the identified analysis requirements can form a starting point for the method. In this endeavour, resources to consult may include among others: the internet, the United States Pharmacopeia (USP), Food and Drug Administration (FDA) requirements, The United States Environmental Protection Agency (EPA) requirements, United States Department of Agriculture (USDA) methods, the Centre for Drug Evaluation and Research (CDER, 1994), the International Union of Pure and Applied Chemistry guidelines, the International Standards Organisation guidelines, the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines, etc (Waters Corporation, 2011).

Selecting Analysis Technique and Conditions

This depends on what type of analysis will provide the information identified in the previous steps. Different techniques provide different capabilities. For example, Mass spectrometry (MS) measures the mass-to-charge ratio of charged particles. Liquid chromatography (LC) on the other hand separates samples in solution based on physical properties such as polarity, ionic strength, and molecular size. The Liquid chromatography-mass spectrometry (LC-MS) technique takes advantage of the physical separation capabilities of LC with the mass analysis capabilities of mass spectrometry. Thermal Analysis analyses materials by utilising the way they change with temperature. Once the analysis technique is selected, initial conditions should be determined. For example, for LC one would select a detector, column, and mobile phase (Waters Corporation, 2011).

Developing the Method

This starts with preparation of samples. For example, for LC select the sample solvent and the proper sample preparation procedures. Method development may be done using one of the approaches: either a stepwise incremental (one-factor-at-a-time) approach based on results from previous experiment or a systematic screening protocol, in which factors such as stationary phases, solvents, pH, and column chemistry are evaluated for selectivity, retention and resolution (Waters Corporation, 2011).

Selecting a Standardisation Technique

If required, a standardisation method should be used. A reference standard is a highly purified compound that is well characterised. Chromatographic methods rely heavily on a reference standard to provide accurate data. Therefore, the quality and purity of the reference standard is very important. Two types of reference standards, chemical and nuclidic, exist. With the latter, the radiolabel purity should also be considered as well as the chemical purity. Chromatographic test methods use either external or internal standards for quantitation (CDER, 1994).

An external standard method is used when the standard is analysed on a separate chromatogram from the sample. Quantitation is based on a comparison of the peak area or height (HPLC or GC) or spot intensity (Thin Layer Chromatography) of the sample to that of a reference standard of the analyte of interest. The external standard method is more appropriate for: Sample with a single target concentration and narrow concentration range, e.g., acceptance and release tests, simple sample preparation procedures and increased baseline time for detection of potential extraneous peaks, e.g., impurities test. The working concentration is the target concentration of the compound of interest as described in the method. Keeping the concentrations of the sample and the standard close to each other for the external standard method improves the accuracy of the method. (CDER, 1994).

With an internal standard method, a compound of known purity that does not cause interference in the analysis is added to the sample mixture. Quantitation is based on the response ratio of compound of interest to the internal standard against the response ratio of a similar preparation of the reference standard (HPLC or GC). This technique is rarely used for TLC methods. The internal standard method is more appropriate for: complex sample preparation procedures, e.g., multiple extractions, low concentration sample (sensitivity being an issue), e.g., pharmacokinetics studies, wide range of concentrations expected in the sample for analysis, e.g., pharmacokinetics studies (CDER, 1994).

Checking the Overall Performance

This can be done by assessing accuracy, precision, reproducibility, linearity, limits of detection and limits of quantitation (Waters Corporation, 2011).

Verification of Method Optimization and Robustness

This can be achieved by using an experimental design approach to determine the experimental factors that have significant impact on the method. For example, HPLC conditions include: % organic, pH, flow rate, temperature, wavelength, column age, MS conditions include: ionisation and mass separation conditions, sample preparation conditions include: % organic, pH, shaking or sonication, sample size, sample age, calculation and standardisation conditions include: integration, wavelength, standard concentration and response factor correction (Waters Corporation, 2011).

Method Validation

The goal of method validation is always to demonstrate that performance results from the method will not be significantly impacted by slight variations of conditions (Waters Corporation, 2011).

METHOD DEVELOPMENT

Chromatographic methods are commonly used for the quantitative and qualitative analysis of raw materials, drug substances, drug products and compounds in biological fluids, based on interaction and differential partition of the sample between the mobile liquid phase and the stationary phase.

The hypothetical protocol described here is generated and approved for validation of the identification and quantitation of Ondansetron hydrochloride dihydrate in Nausetron, Ondansetron hydrochloride 4mg/5ml Syrup by the technique of reversed-phase high performance liquid chromatography (RP-HPLC), according to the hypothetical Standard Operating Procedure (SOP) of JBXY Pharmaceuticals, "Validation of Analytical Test Procedures," SOP Number MUJ-256-B dated 16th July 2015, the French Society for Pharmaceutical Sciences and Techniques (SFSTP 1992), the United States Pharmacopoeia (USP 25), the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and is reported in development report DR213, "Method Development Report for validation of the Identification and Assay of Ondansetron hydrochloride dihydrate in Ondansetron hydrochloride 4mg/5ml Syrup (Nausetron) by Reversed-Phase High Performance Liquid Chromatography". The method is assessed for Robustness, System Suitability, Linearity and Range, Specificity, Accuracy (Recovery), Precision, Limits of Detection and Limit of Quantification (not required for assay method) and Range (Bliesner, D. M. 2006).

Required Materials and Operating Conditions

The required apparatus include a Stuart® magnetic mixer (stirrer), a Wise Clean® ultrasonic bain marie (hot water bath), a Digital Scale, a Thermo Scientific Muffle furnace, a Digital PH-Metre, protective latex gloves and a Thermo Scientific UltiMate™ 3000 Standard Quaternary HPLC System equipped with analytical end capped reversed phase Acclaim™ 120 C18 Columns (25cm x 4.6mm, 5µm) packed with spherical fully porous ultrapure silica substrate stable between pH 2 and 8 and capable of operating at column temperature of 60.0°C at a maximum pressure of 4500 psi and providing a surface area of approximately 300m²/g, a UV-vis detector at a data collection rate of up to 100 Hz and 4 solvent channels with a solvent degasser integrated into the pump module with injection cycle times as low as 15 s and a Dionex Chromeleon® 7 Chromatography Data System. The required glassware includes beakers (1000ml, 100ml and 50ml), graduated cylinders (1000ml and 100ml), vials (50µl), conical flasks (100ml, 50ml and 10ml), graduated pipettes and micropipettes, test tubes and an HPLC syringe.

The required reagents include HPLC Grade ultrapure water, Acetonitrile R solution, 0.02M Monopotassium Phosphate solution (KH_2PO_4), 1M Sodium Hydroxide (NaoH), anhydrous Citric Acid, Sodium Citrate, Sodium Benzoate, 70% crystallisable Sorbitol, Strawberry flavoured Aroma and Ondansetron hydrochloride dihydrate, the active pharmaceutical ingredient (API) in Ondansetron hydrochloride 4mg/5ml syrup provided by JBXY Pharmaceuticals.

The API, Ondansetron hydrochloride as the dihydrate is the racemic form of ondansetron and is a selective blocking agent (selective inhibitor) of the serotonin 5-HT3 receptor type, used in the prevention of nausea and vomiting associated with highly emetogenic cancer chemotherapy, including cisplatin above or equal to 50 mg/m^2 and prevention of postoperative nausea and/or vomiting. Chemically it is (±) 1, 2, 3, 9-tetrahydro-9-methyl-3- [(2-methyl-1H-imidazol-1- yl) methyl]-4H-carbazol-4-one, monohydrochloride, dihydrate (FDA, 2012). It has the structural formula below:

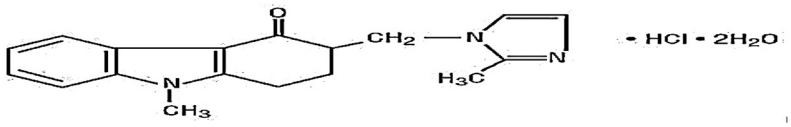

Figure 1: (±) 1, 2, 3, 9-tetrahydro-9-methyl-3- [(2-methyl-1H-imidazol-1- yl) methyl]-4H-carbazol-4-one, monohydrochloride, dihydrate, the racemic form of Ondansetron.

The molecular formula is $C_{18}H_{19}N_3O•HCl•2H_2O$, representing a molecular weight of 365.859. Ondansetron HCl dihydrate is a white to off-white powder that is soluble in water and normal saline. (FDA, 2012)

While its mechanism of action has not been fully characterized, ondansetron is not a dopamine-receptor antagonist. Serotonin receptors of the 5-HT3 type are present both peripherally on vagal nerve terminals and centrally in the chemoreceptor trigger zone of the area postrema. It is not certain whether ondansetron's antiemetic action is mediated centrally, peripherally, or in both sites. However, cytotoxic chemotherapy appears to be associated with release of serotonin from the enterochromaffin cells of the small intestine. In humans, urinary 5-HIAA (5-hydroxyindoleacetic acid) excretion increases after cisplatin administration in parallel with the onset of emesis. The released serotonin may stimulate the vagal afferents through the 5-HT3 receptors and initiate the vomiting reflex (FDA, 2012).

Available research recommends the mobile phase as 50 volumes of acetonitrile R mixed with 50 volumes of 0.02M Monopotassium Phosphate (KH_2PO_4) at pH 5.40. The Acclaim™ 120 C18 Column is to be set at 60°C at a maximum pressure of 4500 psi with a flow rate of 1.5 mL/minute. With the optimum sample volume chosen at 50µl and according to the UV spectra obtained, the wavelength of 216 nm is chosen for quantitative determination.

Safety Precautions

All the necessary precautions should be taken when using each compound. Laboratory safety wear should include a lab coat, safety glasses and safety gloves. Samples and test solutions containing these compounds will be handled, stored, and disposed in accordance with applicable JBXY Pharmaceuticals standard operating procedures, and all applicable state and federal regulations. The safety data sheets for Ondansetron hydrochloride dihydrate and all compounds under the reference material section of the report should be read carefully.

Preparation of Samples

Preparation of Mobile Phase

The mobile phase is prepared by mixing 500ml of 0.02M Monopotassium phosphate buffer solution at PH 5.4 with 500ml of acetonitrile (v/v). These major components are prepared as follows:

With a calibrated digital scale using weights of 200g, 10g and 50mg, a 0.02M Monopotassium phosphate solution is prepared by accurately weighing 2.718 g of powdered KH_2PO_4 and depositing it in a 100ml conical flask. Without completing to the volume mark, a small amount of HPLC grade ultrapure water is added to dissolve the powder. In parallel, a minor component, 1M Sodium hydroxide solution is prepared by accurately weighing 4.20g of solid NaoH and depositing it in a 100ml conical flask and then dissolving it with HPLC grade ultrapure water to the gauge mark.

Using an already calibrated digital pH-Metre with standard solutions of pH4 and PH7, the pH of the prepared KH_2PO_4 solution is adjusted drop by drop to 5.4 using the prepared 1 M NaOH solution, the whole time mixing and stirring with a Stuart® magnetic mixer (stirrer). Once a pH of 5.4 is attained, the flask containing the KH_2PO_4 solution is brought to volume with ultrapure water to form a buffer solution containing 0.02M Monopotassium phosphate.

500ml of the prepared buffer solution is transferred into a 1000ml beaker and 500ml of acetonitrile added up to the gauge mark. The constituted mobile phase is degassed for 5 minutes in a Wise Clean® ultrasonic bath equipped with a vacuum system and the beaker labelled "MOBILE PHASE" (Boudis H, 2015).

Preparation of Calibration Standard

According to the United States Pharmacopeia 25 (USP 25), a concentration interval of 80% to 120% in API is recommended for method development involving pharmaceutical drug substances and drug products. The calibration standard is a series of solutions containing an increasing concentration of Ondansetron hydrochloride dihydrate alone without the matrix (excipients) of the syrup. It is prepared as follows: A stock solution containing Ondansetron hydrochloride dihydrate alone without matrix is prepared by accurately weighing 90mg of the active ingredient and placing it in a 100ml conical flask. A small volume of the already prepared mobile phase is added for dissolution and after a brief sonication, the volume is finally brought progressively to the mark with the mobile phase to form a stock solution corresponding to the concentration of 90µg/ml. The flask is marked "**STOCK ETALON**" solution.

On each of the 3 non-consecutive days, 8, 9, 10, 11 and 12 millilitres are pipetted from the prepared **STOCK ETALON** solution and each deposited in a different 100ml volumetric flask, to which the prepared mobile phase is progressively added to volume with constant stirring to form diluted solutions of concentration: 72, 81, 90, 99 and 108mg/l, corresponding to a percentage concentration level of 80, 90, 100, 110 and 120% respectively. These flasks are marked: **ETALON A 80%, ETALON A 90%, ETALON A 100%, ETALON A 110% and ETALON A 120%** respectively and

represent the series of calibration standards (Boudis H, 2015).

Preparation of Matrix (Placebo)

The Syrup matrix (excipients alone) is prepared by accurately weighing 0.6g of Anhydrous citric acid, 0.3g of Sodium citrate, 0.3g of Sodium benzoate, 60g of 70% crystallisable Sorbitol, 100mg of strawberry flavour and depositing all these into a 100ml conical flask. The flask is brought to mark with HPLC grade ultrapure water and the mixture homogenised with a Stuart® magnetic stirrer. The flask is marked "STOCK PLACEBO". 9 millilitres then are pipetted from the STOCK PLACEBO solution into a 100ml volumetric flask and the flask brought to volume with the mobile phase and this sample marked "**PLACEBO 100%**" (Boudis H, 2015).

Preparation of Validation Standard

The validation standard is a series of reconstituted dosage form samples (syrup) containing increasing concentration of Ondansetron hydrochloride dihydrate mixed with excipients (matrix). It is thus the same as a spiked placebo. This series is prepared as follows. A Stock Syrup is reconstituted by accurately weighing 100mg of Ondansetron hydrochloride dihydrate powder (not 90mg but 100mg since the API exists as dihydrate in the dosage form) and depositing it in a 100ml flask to which accurately measured components of the matrix are added in quantities 0.6g of Anhydrous citric acid, 0.3g of Sodium citrate, 0.3g of Sodium benzoate, 60g of 70% crystallisable Sorbitol, 100mg of strawberry flavour and 100ml of HPLC grade ultrapure water and the flask marked "**STOCK PA+EXCIPIENTS**". On each of the 3 non-consecutive days, 7.2, 8.1, 9.0, 9.9 and 10.8 millilitres are pipetted from the prepared **STOCK PA+EXCIPIENTS** solution and each deposited in a different 100ml volumetric flask to which the prepared mobile phase is progressively added to the volume mark with constant stirring to form diluted solutions of concentration: 72, 81, 90, 99 and 108mg/l, corresponding to a percentage concentration level of 80, 90, 100, 110 and 120% respectively and marked **PA+EXCIPIENTS A 80%, PA+EXCIPIENTS A 90%, PA+EXCIPIENTS A 100%, PA+EXCIPIENTS A 110%** and **PA+EXCIPIENTS A 120%** respectively (Boudis H, 2015).

Preparation of System Suitability Samples

On each of the 3 days, from the prepared syrup at 100% (PA+EXCIPIENTS A 100%), 6 system suitability samples are prepared by transferring exactly 50µl to each of the 6 vials This procedure is repeated for 3 non-consecutive days; once each day. The three tables below show: instrument calibration and measurement values for PH and weight of Monopotassium phosphate and Sodium hydroxide, expected and observed measurements of Ondansetron hydrochloride dihydrate for the calibration standards and the expected and observed measurements for the components of stock reconstituted syrup and stock placebo taken on the 3 days.

Table 1: Instrument calibration and measurement values for PH and weight of Monopotassium phosphate and Sodium hydroxide for the 3 days.

(Boudis s.d.)	**Instrument calibration values**				**Measurement values**					
	Digital Balance (g)		**PH-Metre**		**KH_2PO_4(g)**		**NaOH(g)**		**PH**	
Day	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**	**Observed**	**Before**	**After**
1	200.000	**200.000**			2.7218	**2.7219**	4.20	**4.19**	4.63	**5.40**
	10.0000	**09.9999**	7.00	**7.04**						
	0.05000	**0.04998**	4.00	**4.02**						
2	200.000	**200.000**			2.7218	**2.7218**	4.20	**4.20**	4.57	**5.42**
	10.0000	**09.9999**	7.00	**7.01**						
	0.05000	**0.04998**	4.00	**4.00**						
3	200.000	**200.000**			2.7218	**2.7218**	4.20	**4.20**	4.56	**5.41**
	10.0000	**09.9999**	7.00	**7.02**						
	0.05000	**0.04999**	4.00	**4.00**						

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Table 2: Expected and observed measurements of Ondansetron hydrochloride dihydrate for the calibration standards on the 3 days

	Ondansetron hydrochloride dihydrate	
Day	**Expected (mg)**	**Observed (mg)**
1	90.00	**90.10**
2	90.00	**90.20**
3	90.00	**90.50**

Table 3: Expected and observed measurements for the components of stock reconstituted syrup and stock placebo taken on the 3 days.

		STOCK PA+EXCIPIENTS (mg/100ml)		STOCK PLACEBO (mg/100ml)	
Component	**Day**	**Expected**	**Observed**	**Expected**	**Observed**
Ondansetron hydrochloride dihydrate (API)	**1**	100.00	100.30	00.00	00.00
	2	100.00	100.60	00.00	00.00
	3	100.00	100.10	00.00	00.00
Excipients (mg/100ml)					
Anhydrous Citric acid	**1**	600.00	601.10	600.00	601.10
	2	600.00	600.10	600.00	600.10
	3	600.00	600.10	600.00	600.10
Sodium citrate	**1**	300.00	301.30	300.00	301.30
	2	300.00	300.20	300.00	300.20
	3	300.00	300.20	300.00	300.20
Sodium benzoate	**1**	300.00	301.30	300.00	301.30
	2	300.00	300.20	300.00	300.20
	3	300.00	300.20	300.00	300.20
70% Crystallisable sorbitol	**1**	6000.00	6020.80	6000.00	6020.80
	2	6000.00	6010.60	6000.00	6010.60
	3	6000.00	6010.60	6000.00	6010.60
Strawberry flavour	**1**	100.00	100.40	100.00	100.40
	2	100.00	100.30	100.00	100.30
	3	100.00	100.20	100.00	100.20

METHOD VALIDATION

Throughout the regulatory submission process, the validation of methods may be required to demonstrate the scientific soundness of the measurement employed (Boudis H, 2015). The validation practice demonstrates that an analytic method measures the correct target substance, in the correct amount, and in the appropriate range for the intended samples. It allows the analyst to comprehend the behaviour of the method and to establish its performance limits.

This part of the paper illustrates how the obtained data on the variation of the concentration of API with the obtained response signal is mathematically treated to determine if validation acceptance criteria are met. A detailed study of parameters such as robustness, system suitability, linearity, specificity, accuracy, precision, limit of detection, Limit of Quantification, Sensitivity and Range is given (Boudis H, 2015).

The Study of Robustness and System Suitability

Robustness (Ruggedness)

The robustness (ruggedness) of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters. It provides an indication of method reliability during normal usage. Robustness can therefore be described as the ability to reproduce the (analytical) method in different laboratories or under different circumstances without the occurrence of unexpected differences in the obtained result. The ICH guidelines recommend that one consequence of the evaluation of robustness should be that a series of system suitability parameters (such as resolution tests) is established to ensure that the validity of the analytical procedure is maintained whenever used (Vander H, et al, 2001).

Robustness tests were originally introduced to avoid problems in interlaboratory studies and to identify the potentially responsible factors (Youden, W. J, 1975). This means that a robustness test was performed at a late stage in the method validation since interlaboratory studies are performed in the final stage. Thus, the robustness test was considered a part of method validation related to the precision (reproducibility) (Van Leeuwen, et al, 1991). However, performing a robustness test late in the validation procedure involves the risk that when a method is found not to be robust, it should be redeveloped and optimised. At this stage much effort and money have already been spent in the optimisation and validation. In order to avoid this, the performance of a robustness test has been shifting to earlier points of time in the life of the method.

The robustness test examines the potential sources of variability in one or a number of responses of the method for which system suitability test limits can be defined (e.g. resolution, tailing factors, capacity factors, column efficiency in a chromatographic method). To examine potential sources of variability, a number of factors are selected from the operating procedure and examined in an interval that slightly exceeds the variations which can be expected when a method is transferred from one instrument to another or from one laboratory to another. These factors are then examined in an experimental design and the effect of the factors on the response (s) of the method

is evaluated. In this way the factors that could impair the method performance are discovered. The analyst then knows that such factors must be more strictly controlled during the execution of the method. The information gained from the robustness test can be used to define System Suitability Limits, based on experimental evidence and not arbitrarily on the experience of the analyst (Dadgar, D., et al, 1995). In the case of this method for example, these factors include:

Mobile Phase Variation

Each major component is alternatingly increased and decreased by 5% and 10%, and at each level of increase and decrease, 50µl of the system suitability standard are injected 6 times and the appropriate figures of merit measured. Each minor component (less than 10ml/l) is alternatingly increased and decreased 15% and 30% and at each level of increase and decrease, 50µl of the system suitability standard are injected 6 times and the appropriate figures of merit measured. (Bliesner, D. M. 2006).

HPLC Column Temperature Variation Using a Column Heater

The system suitability standard is injected 6 times at 60°C then at 65°C and finally at 55°C, each time measuring the appropriate figures of merit.

Mobile Phase Flow-Rate Variation

The system suitability standard is injected 6 times at 10% increase in flow rate then at 10% decrease, then at 25% increase and finally at 25% decrease, each time measuring the appropriate figures of merit.

For Buffer pH Variation

The system suitability standard is injected 6 times at 0.25 pH units increase followed by an injection at 0.25 pH units decrease, each time measuring the appropriate figures of merit (Dadgar, D., et al, 1995)

HPLC Column Variation

The system suitability standard is injected 6 times in each column, using three columns from at least two different lots of packing material and the appropriate figures of merit measured. The system suitability standard is then injected 6 times using a brand-new column and an old column that has done more than 500 injections. The figures of merit are determined.

Acceptance Criteria

Measured figures of merit include: resolution, tailing factor, theoretical plates, and capacity factor for each variation experiment. The suitability of the method is determined under each modification by taking into account peak shape, retention time, system pressure, and system suitability parameters. System suitability parameters important to the overall function of the method are identified and limits for critical parameters established. For column variability, it should be ensured that all columns used in validation are commercially available. It should also be ensured that three columns from at least two different lots of packing material are obtained and used as well as a brand-new column and an old column (more than 500 injections) and that the retention times are similar on each of the three columns (Bliesner, D. M. 2006).

System Suitability Specifications

System suitability is the evaluation of the components of an analytical system to show that the performance of the system meets the standards required by a given method. A suitability evaluation usually contains its own set of parameters; for chromatographic assays, these may include tailing factors, resolution, and precision of standard peak areas, and comparison to a confirmation standard, capacity factors, retention times, theoretical plates, and calibration curve linearity. Where applicable, system suitability parameters are calculated, recorded, and trended throughout the course of the validation. Final values are then determined from this history.

Characteristics of a Chromatographic Peak

An ideal elution peak can be approximated to a Normal distribution (Gaussian) curve with mean ($\boldsymbol{\mu}$) equal to the retention time and standard deviation ($\boldsymbol{\sigma}$) (Roussac. et al, 1997). The response signal ($\boldsymbol{y}$) is a probability density function of time ($\boldsymbol{x}$) at the detector located at the column outlet. This function describes an even curve (maximum for $\boldsymbol{x = 0}$, $y = 0.399$) which has two points of inflexion for $\boldsymbol{x = \pm 1}$, at $y = 0.242$, 60.6% of maximum height from the base and whose width at the points of inflection is equal to $\boldsymbol{2\sigma}$.

In chromatography, ($\boldsymbol{\delta}$) denotes the width at half maximum height ($\boldsymbol{\delta = 2.35\sigma}$) and $\boldsymbol{\sigma^2}$ the variance of the peak. The width at the base of the peak ($\boldsymbol{\omega}$) is measured at 13.5% of the height and ($\boldsymbol{\omega = 4\sigma}$) at which the point the curve is considered Gaussian. It can be deduced that 95.4% of the total area under the curve is within 4 standard deviations between -2 and +2. The un even distribution of analyte concentration in the deposition zone at the top of the column and the fact that the speed of the mobile phase is zero at the walls and maximum at the centre of the column means that actual chromatograms sometimes are far from having Gaussian-like peaks. The symmetry of the observed peak is translated by two parameters;

factor of asymmetry ($\boldsymbol{F_a}$) and Tailing factor ($\boldsymbol{F_t}$), measured at 10% of maximum height.

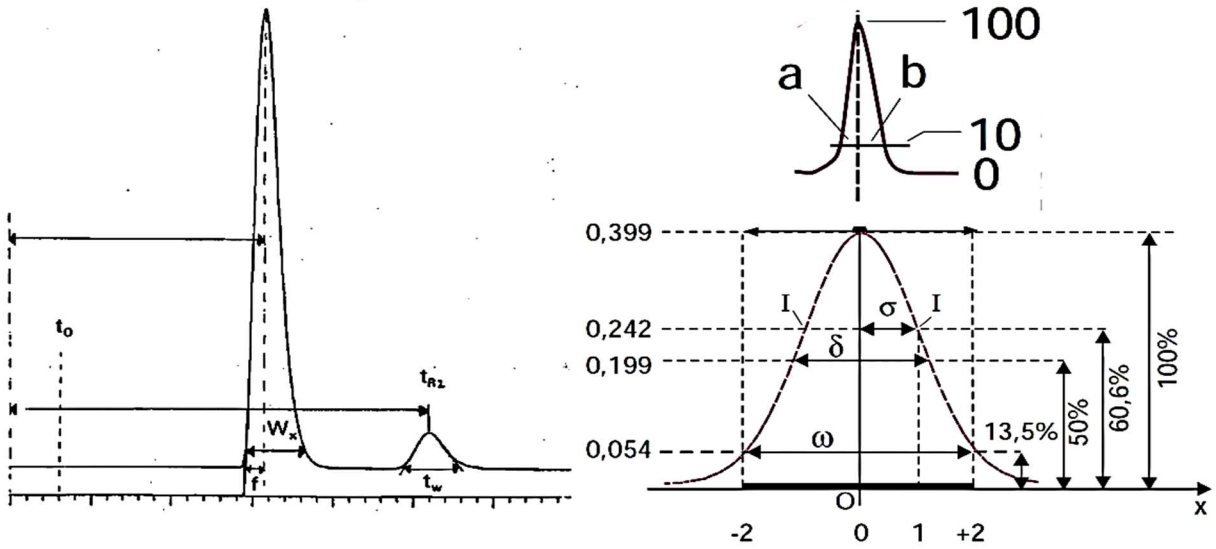


Figure 2: Definition of Terms for the System Suitability Parameters using the Chromatogram Peak.

Where;
$\boldsymbol{\omega = 1.7\delta = 4\sigma}$ = width of the peak determined at about 13,5% from the baseline of the peak height.
$\boldsymbol{\delta = 2.35\sigma}$ = width of the peak determined at about 50% from the baseline of the peak height.
$\boldsymbol{\sigma}$ =standard deviation from the mean of retention time equivalent to half the width of the peak determined at 60% from the baseline of the peak height.
$\boldsymbol{W_x = a + b}$ = width of the peak determined at about 10% from the baseline of the peak height.
$\boldsymbol{f = a}$ = distance between peak maximum and peak front at $\boldsymbol{W_x}$.
$\boldsymbol{t_0}$ = elution time of the void volume or non-retained components.
$\boldsymbol{t_R}$ = retention time of the analyte.
$\boldsymbol{t_W}$= peak width measured at baseline of the extrapolated straight sides to baseline.

In our case, to determine these parameters, on each of the 3 days, 6 injections of 50µl each from the system suitability samples are made and the results treated as follows:

Precision (injection repeatability)

Although sample preparation and manufacturing variations are not accounted for, injection precision expressed as relative standard deviation (RSD) or variation coefficient indicates the performance of the high-performance liquid chromatograph which includes the plumbing, column and environmental conditions at the time the samples are analysed. RSD values less than or equal to 1% for 5 or more injections are desired. For the 6 injections made from the system suitability samples, RSD values less than 1% were obtained on each of the 3 days for this method development.

Retention Factor or Capacity factor (k')

$$k' = \frac{(t_R - t_0)}{t_0}$$

The capacity factor is a measure of where the peak of interest is located with respect to the elution time of the non-retained components (void volume). The Centre for Drug Evaluation and Research recommends k' values generally greater than 2 as an indication that the peak is well resolved from other peaks and the void volume. A capacity factor greater than 2 was obtained for all system suitability samples on each of the 3 days.

Relative retention ($\boldsymbol{\alpha}$)

$$\alpha = \frac{k_1'}{k_2'}$$

Relative retention is a measure of the relative location of two peaks. This is not an essential parameter as long as the resolution (R_s) is stated.

Resolution ($\boldsymbol{R_s}$)

$$R_s = \frac{t_{R_2} - t_{R_1}}{\frac{1}{2}(t_{W_2} + t_{W_1})} = 1.177\left(\frac{t_{R_2} - t_{R_1}}{\delta_2 + \delta_1}\right)$$

The resolution is a measure of how well two peaks are separated, especially for quantitation. This is a very useful parameter if potential interference peak(s) may be of concern. R_s values greater than 2 between the peak of interest and the closest potential interfering

peak (impurity, excipient, degradation product, internal standard, etc.) are desirable. A resolution above 2 was obtained for all system suitability samples on each of the 3 days between the peak for Sodium benzoate and that of Ondansetron hydrochloride dihydrate.

Asymmetry $(\boldsymbol{F_a})$ *and Tailing Factor* $(\boldsymbol{F_t})$

$$F_a = \frac{b}{a} = \frac{\boldsymbol{W_x - a}}{a}$$

$$F_t = \frac{1}{2}\left(\frac{a+b}{a}\right) = \frac{1}{2}\left(\frac{\boldsymbol{W_x}}{a}\right)$$

The accuracy of quantitation decreases with increase in peak tailing because of the difficulties encountered by the integrator in determining where and when the peak ends and hence the calculation of the area under the peak. Integrator variables are pre-set by the analyst for optimum calculation of the area for the peak of interest. Tailing factor values less than or equal to 2 are recommended. A mean tailing factor less than 2 was obtained for all system suitability samples on each of the 3 days.

Theoretical Plate Number $(\boldsymbol{N})$ *or Theoretical Column Efficiency*

$$\boldsymbol{N} = \frac{\boldsymbol{L}}{\boldsymbol{HEPT}} = 16\left(\frac{t_R^2}{t_W^2}\right) \approx 16\left(\frac{t_R^2}{\boldsymbol{\omega^2}}\right) \approx 5.54\left(\frac{t_R^2}{\boldsymbol{\delta^2}}\right)$$

Theoretical plate number is a measure of column efficiency, that is, how many peaks can be located per unit run-time of the chromatogram. $\boldsymbol{N}$ is fairly constant for each peak on a chromatogram with a fixed set of operating conditions. $\boldsymbol{H}$, or $\boldsymbol{HEPT}$, the height equivalent of a theoretical plate, measures the column efficiency per unit length $(\boldsymbol{L})$ of the column. Parameters which can affect $\boldsymbol{N}$ or $\boldsymbol{H}$ include peak position, particle size in column, flow-rate of mobile phase, column temperature, viscosity of mobile phase, and molecular weight of the analyte. The theoretical plate number depends on elution time but in general should be greater than 2000. $\boldsymbol{N}$ values greater than 2000 were obtained for all system suitability samples on each of the 3 days. The table below shows peak characteristics obtained from system suitability testing on each of the 3 days.

Table 4: Peak characteristics obtained from system suitability testing on each of the 3 days.

Day	Inj	System Suitability Sample	t_R (min)	Area (mAUmin)	Height (mAU)	Width δ (50%) (min)	Asym (T_t)	Plates (N)
1	1	PA+EXCIPIENTS A 100%	3.618	233.557	2411.14	0.088	1.57	9275
	2	PA+EXCIPIENTS A 100%	3.617	223.777	2431.82	0.088	1.59	9421
	3	PA+EXCIPIENTS A 100%	3.620	233.806	2448.19	0.087	1.59	9572
	4	PA+EXCIPIENTS A 100%	3.623	233.816	2426.06	0.088	1.57	9408
	5	PA+EXCIPIENTS A 100%	3.622	233.929	2445.93	0.087	1.57	9555
	6	PA+EXCIPIENTS A 100%	3.623	233.986	2446.00	0.087	1.60	9566
		Max	**3.623**	**233.986**	**2448.86**	**0.088**	**1.60**	**9572**
		Mean	**3.621**	**233.811**	**2434.86**	**0.088**	**1.58**	**9466**
		Min	**3.617**	**233.557**	**2411.14**	**0.087**	**1.57**	**9275**
		SD	**0.0026**	**4.1022**	**14.6537**	**0.0005**	**0.0133**	**119.18**
		RSD (%)	**0.0715**	**1.7545**	**0.6018**	**0.6224**	**0.8412**	**1.2591**
2	1	PA+EXCIPIENTS A 100%	3.618	233.858	2448.043	0.088	1.58	9349
	2	PA+EXCIPIENTS A 100%	3.618	233.781	2447.525	0.088	1.59	9334
	3	PA+EXCIPIENTS A 100%	3.618	233.731	2454.026	0.089	1.59	9154
	4	PA+EXCIPIENTS A 100%	3.617	233.719	2481.192	0.088	1.58	9296
	5	PA+EXCIPIENTS A 100%	3.615	233.892	2478.433	0.089	1.60	9236
	6	PA+EXCIPIENTS A 100%	3.620	233.699	2444.023	0.090	1.59	9041
		Max	**3.620**	**233.892**	**2481.192**	**0.090**	**1.60**	**9349**
		Mean	**3.618**	**233.780**	**2458.873**	**0.089**	**1.59**	**9235**
		Min	**3.615**	**233.699**	**2444.023**	**0.088**	**1.58**	**9041**
		SD	**0.0016**	**0.0791**	**16.5579**	**0.0008**	**0.0075**	**118.92**
		RSD (%)	**0.0451**	**0.0339**	**0.6734**	**0.9174**	**0.4734**	**1.2878**
3	1	PA+EXCIPIENTS A 100%	3.620	233.931	2462.744	0.087	1.56	9561
	2	PA+EXCIPIENTS A 100%	3.620	233.759	2455.495	0.087	1.56	9616
	3	PA+EXCIPIENTS A 100%	3.621	233.743	2470.484	0.087	1.55	9608
	4	PA+EXCIPIENTS A 100%	3.617	233.726	2457.741	0.086	1.56	9711
	5	PA+EXCIPIENTS A 100%	3.623	233.757	2457.042	0.087	1.56	9604
	6	PA+EXCIPIENTS A 100%	3.621	233.760	2458.557	0.087	1.56	9653
		Max	**3.623**	**233.931**	**2470.484**	**0.087**	**1.56**	**9711**
		Mean	**3.620**	**233.779**	**2460.344**	**0.087**	**1.56**	**9625**
		Min	**3.617**	**233.726**	**2455.495**	**0.086**	**1.55**	**9561**
		SD	**0.0020**	**0.0754**	**5.5311**	**0.0004**	**0.0041**	**51.157**
		RSD (%)	**0.0543**	**0.0323**	**0.2248**	**0.4693**	**0.2617**	**0.5315**

All relative standard deviations are less than 2%.

The Study of Linearity

Within a clearly defined range, linearity evaluates the ability of the method to obtain a response signal that is directly proportional to the concentration of analyte standard. If the method is linear, the test results are directly, or by well-defined mathematical transformations, proportional to the concentration of analyte in samples within a given range. Note that this is different from range (sometimes referred to as method linearity), which is evaluated using samples and must encompass the specification range of the component assayed in the drug product. It can be established for all active substances, preservatives, and expected impurities. Evaluation is performed on standards (Bliesner, D. M. 2006).

For every level of API of the calibration standard (ETALON), and for every level of API concentration of the validation standard (PA+EXCIPIENTS), 1 injection of 50µl is made in the HPLC system on each of the 3 non-consecutive days and the response obtained recorded. It should be ensured that sample injection is made from the lowest concentration to the highest concentration to reduce the effects, if any, of carryover from the higher concentration samples. The table below shows the response signal obtained in the two cases.

Table 5: Response Signals obtained after injection of increasing levels of concentration of API alone and API in the reconstituted dosage form on each of the 3 days for this method validation.

Level	Day	API Alone (ETALON)			Dosage Form (PA+EXCIPIENTS)		
		API Conc. (mg)	Concentration (%)	Signal (mAU)	API Conc. (mg)	Concentration (%)	Signal
Group 1 (80%)	**1**	72.08	80.09	1856.14	72.08	80.09	1851.09
	2	72.16	80.18	1833.31	72.16	80.18	1878.98
	3	72.40	80.45	1825.14	72.40	80.45	1817.84
Group 2 (90%)	**1**	81.09	90.10	2004.49	81.09	90.10	2060.16
	2	81.18	90.20	2064.84	81.18	90.20	2098.20
	3	81.45	90.50	2053.83	81.45	90.50	2105.88
Group 3 (100%)	**1**	90.10	100.11	2260.09	90.10	100.11	2330.11
	2	90.20	100.22	2241.32	90.20	100.22	2454.19
	3	90.50	100.55	2217.37	90.50	100.55	2384.28
Group 4 (110%)	**1**	99.11	110.12	2507.07	99.11	110.12	2567.26
	2	99.22	110.24	2493.05	99.22	110.24	2501.79
	3	99.55	110.60	2398.53	99.55	110.60	2572.94
Group 5 (120%)	**1**	108.12	120.13	2762.17	108.12	120.13	2819.11
	2	108.24	120.26	2784.32	108.24	120.26	2859.80
	3	108.60	120.65	2719.79	108.60	120.65	2902.95

Determination of the Regression Model

This is a mathematical function that describes the relationship between the analyte concentration and the response signal. Imagine that it were possible to analyse all API concentrations in the universe in a way that $(\boldsymbol{c})$ distinct levels each marked $(\boldsymbol{l_i})$ in percentage concentration of API are prepared and an infinite number $(\boldsymbol{p})$ of API concentrations in mg/l each marked $(\boldsymbol{x_i})$ from these levels is injected and each corresponding detected response signal marked $(\boldsymbol{y_i})$ is associated with a predictable or expected value $(\boldsymbol{E(y_i)})$, with $(\overline{\boldsymbol{y}}_{\boldsymbol{i}})$ the mean of all response signals. If in order to evenly distribute individual errors $(\boldsymbol{\varepsilon_i})$ that may be due to measurement and the like and to show that the collected data is consistent over time, imagine that this procedure were repeated an infinite number of non-consecutive days such that each distinct level of API percentage concentration corresponds to all the response signals obtained on the different days for that level and $(\overline{\boldsymbol{Y}}_{\boldsymbol{i}})$ is their local mean with $(\boldsymbol{Y_{ij}})$ the response signal corresponding to each individual $\boldsymbol{x_i}$. If $(\boldsymbol{a})$ and $(\boldsymbol{b})$ represent the slope and intercept of the line, among all possible lines, that has the smallest sum of squared vertical differences between $(\boldsymbol{y_i})$ and $(\boldsymbol{E(y_i)})$, then each individual observed response signal would be represented by the equation:

$$\boldsymbol{y_i = ax_i + b + \varepsilon_i}$$

The expected value of this function is the mean of the distribution:

$$\boldsymbol{E(y_i) = E(ax_i) + E(b) + E(\varepsilon_i)}$$

If the errors are assumed to be normally distributed with mean $\mu = 0$ and standard deviation $\sigma = 1$, it follows that $\boldsymbol{\varepsilon_i = 0}$ and the population regression model would be a 2-parameter linear function:

$$\boldsymbol{E(y_i) = ax_i + b}$$

Although this model assumes that using both the population regression slope and population regression intercept yields better estimates of the unknown API concentrations, when restricted to only the intercept, a 1-factor model that assumes that knowing the expected response population signal at

0 API concentration is sufficient enough to estimate unknown API concentrations is obtained.

$$\boldsymbol{E(y_i) = \bar{y}_i}$$

However, since it is impossible to achieve these population conditions in real life, we can take a representative sample from this population and use the sample's data (like the one collected for this current method validation) to approximate the unobservable population slope and intercept using sample slope estimate $(\hat{a})$ and sample intercept estimate $(\hat{b})$, based on the observable values of x_i and y_i. In this case, each individual sample response signal would be associated with a predicted value $(\hat{y}_i)$ and a Residual Error (e_i).

$$\boldsymbol{y_i = \hat{a}x_i + \hat{b} + e_i}$$

Assumed to be normally distributed with mean $\mu = 0$ and standard deviation S_{yx} and having constant variance for all sample response signals at all levels of API concentration (Homoscedasticity), the deviations in the observed response signal are also assumed to be independent over time. It follows that $e_i = 0$ and the regression model (Unrestricted) for this sample is a linear 2-parameter function:

$$\boldsymbol{\hat{y}_i = \hat{a}x_i + \hat{b}}$$

The Restricted regression model is:

$$\boldsymbol{\hat{y}_i = \bar{y}_i}$$

The sample regression slope $(\hat{a})$ can thus be determined as the ratio of $(SSxy)$ to $(SSx$ where; $SSxy$ is the sum of the product of the difference between each API concentration and the mean of concentration and the difference between each observed response signal and the mean of (n) observed response signals and $SSxx$ is the sum of the squared difference between each API concentration and the mean of concentration. The sample regression intercept $(\hat{b})$ is determined as the difference between the mean observed sample response signal and the product of the mean sample concentration and the sample regression gradient.

$$\boldsymbol{\hat{a} = \frac{SSxy}{SSxx}}$$

$$\boldsymbol{\hat{a} = \frac{\sum_{i=1}^{i=n}(x_i - \bar{x}_i)(y_i - \bar{y}_i)}{\sum_{i=1}^{i=n}(x_i - \bar{x}_i)^2}}$$

Where;

$$\boldsymbol{\bar{x}_i = \frac{\sum_{i=1}^{i=n} x_i}{n}}$$

and

$$\boldsymbol{\bar{y}_i = \frac{\sum_{i=1}^{i=n} y_i}{n}}$$

The sample regression intercept can be got from:

$$\boldsymbol{\hat{b} = \bar{y}_i - \hat{a}\bar{x}_i}$$

The table below shows the determination of sample regression slope and sample regression intercept for the response signal from samples of API alone and samples of the reconstituted syrup.

Table 6: Determination of sample regression slope and sample regression intercept for the response signal from samples of API alone and samples of the reconstituted syrup.

Level	Day	API Alone (ETALON)				Dosage Form (PA+EXCIPIENTS)			
		API Conc. (mg)	Response Signal (mAU)	API conc. and Response Signal Co-variability	API Conc. Variability	API Conc. (mg)	Response Signal (mAU)	API conc. and Response Signal Co-variability	API Conc. Variability
Group		x_i	y_i	$(x_i - \bar{x}_i)(y_i - \bar{y}_i)$	$(x_i - \bar{x}_i)^2$	x_i	y_i	$(x_i - \bar{x}_i)(y_i - \bar{y}_i)$	$(x_i - \bar{x}_i)^2$
Group 1 (80%)	1	72.10	1856.14	7.49E+03	3.30E+02	72.10	1851.09	9.01E+03	3.30E+02
	2	72.20	1833.31	7.86E+03	3.27E+02	72.20	1878.98	8.46E+03	3.27E+02
	3	72.40	1825.14	7.92E+03	3.19E+02	72.40	1817.84	9.46E+03	3.19E+02
Group 2 (90%)	1	81.10	2004.49	2.42E+03	8.41E+01	81.10	2060.16	2.63E+03	8.41E+01
	2	81.20	2064.84	1.84E+03	8.23E+01	81.20	2098.20	2.26E+03	8.23E+01
	3	81.50	2053.83	1.88E+03	7.69E+01	81.50	2105.88	2.11E+03	7.69E+01
Group 3 (100%)	1	90.10	2260.09	1.36E+00	2.89E-02	90.10	2330.11	2.87E+00	2.89E-02
	2	90.20	2241.32	1.87E+00	4.90E-03	90.20	2454.19	-7.51E+00	4.90E-03
	3	90.50	2217.37	-1.17E+01	5.29E-02	90.50	2384.28	8.58E+00	5.29E-02
Group 4 (110%)	1	99.10	2507.07	2.11E+03	7.80E+01	99.10	2567.26	1.95E+03	7.80E+01
	2	99.20	2493.05	2.01E+03	7.97E+01	99.20	2501.79	1.38E+03	7.97E+01
	3	99.60	2398.53	1.22E+03	8.70E+01	99.60	2572.94	2.11E+03	8.70E+01
Group 5 (120%)	1	108.10	2762.17	8.81E+03	3.18E+02	108.10	2819.11	8.42E+03	3.18E+02
	2	108.20	2784.32	9.26E+03	3.21E+02	108.20	2859.80	9.20E+03	3.21E+02
	3	108.60	2719.79	8.28E+03	3.36E+02	108.60	2902.95	1.02E+04	3.36E+02
		$\bar{x}_t$	$\bar{y}_t$	SSxy	SSxx	$\bar{x}_t$	$\bar{y}_t$	SSxy	SSxx
		90.27	2268.10	6.11E+04	2.44E+03	90.27	2346.97	6.72E+04	2.44E+03
SLOPE: $\hat{a}$		25.0090				27.5025			
INTERCEPT: $\hat{b}$		10.6183				-135.5877			
MODEL: $\hat{y}$		$\hat{y}_i = 25.0090x_i + 10.6183$				$\hat{y}_i = 27.5332x_i - 138.5374$			

From the table above, the two-factor sample regression models obtained are:

$$\hat{y}_i = 25.0090x_i + 10.6183$$

For the API alone and;

$$\hat{y}_i = 27.502 \quad _i - 135.5877$$

For the API in the reconstituted syrup.

Validity of the Unrestricted Model: $\hat{y}_i = \hat{a}x_i + \hat{b}$

Since Ordinary Least Squares (OLS) estimates are chosen to minimize the sum of squared residuals, the Residual Sum of Squares never decreases (and generally increases) when certain restrictions (such as dropping variables) are introduced into the model (Uriel, E. 2013). When the 2-factor regression model $(k = 2)$ is restricted to an intercept-only model $(q = 1)$, an α- confidence level Fisher-test can be performed to calculate a statistic under the null hypothesis which is distributed as a Snedecor's F random variable with $(q, n - k)$ degrees of freedom for which the probability of occurring under the null hypothesis is equal to the $\rho - value$.

$$F \setminus H_0 \sim F^{\alpha}_{q,n-k}$$

Therefore, to prove that the Unrestricted Model is justified over the Restricted one, it is sufficient to show that restricted model increases residual errors, the unrestricted model explains the residual variance and that the unrestricted model fits well and there is no lack of fit. All these prove that the slope is statistically significant in the Unrestricted Model. The appropriate null and alternate hypotheses are specified at $\alpha = 0.05$ and rejection and non-rejection regions of the null hypothesis are defined as shown in the figure below.

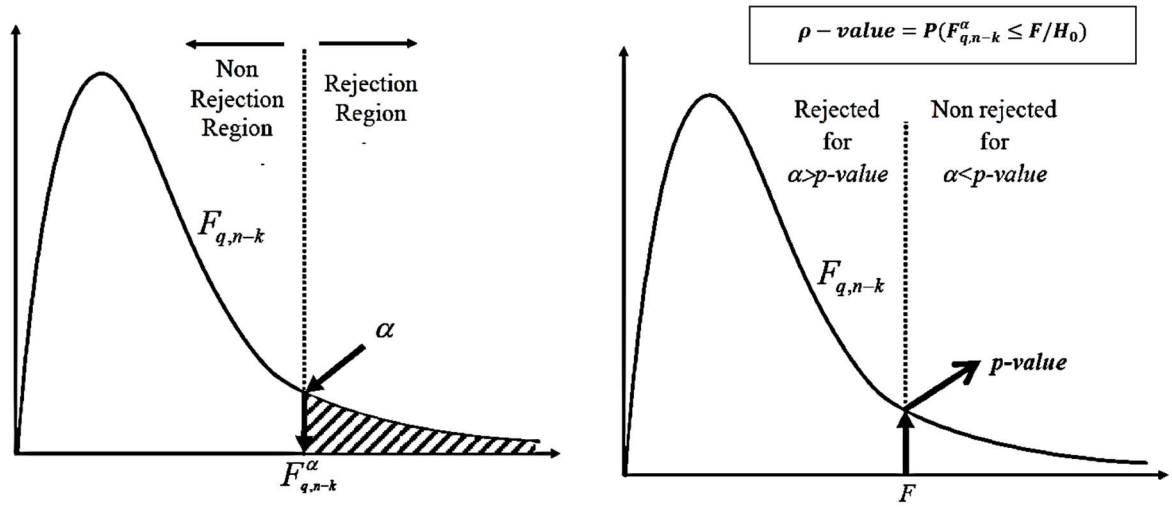


Figure 3: Rejection and non-Rejection regions using F-distribution and p-values using the F-distribution.

Proof of Residual Variance Increase in the Restricted Model: $\hat{y}_i = \bar{y}_i$

The increase in the residual variance in the restricted model can be assessed by using the R-squared form of the F-test based on the R-squared values of the models or using the ratio of the difference in the residual variance of the models. The variability of the response signal can be measured using three sums of squares; **SST**, **SSR** and **SSE**. The Total Sum of Squares (**SST**) is proportional to the variance of the data. The variance of the data is the mean total sum of squares and is distributed with $(n - 1)$ degrees of freedom (df_T).

$$SST = SSYY = \sum_{i=1}^{i=n} (y_i - \bar{y}_i)^2, df_T = n - 1$$

The Regression Sum of Squares (**SSR**) is the variation that is explained by the chosen regression model. The mean of the regression sum of squares represents the variance explained by the adopted model and it is distributed with (1) degree of freedom (df_R).

$$SSR = \sum_{i=1}^{i=n} (\hat{y}_i - \bar{y}_i)^2, df_R = 1$$

The Error Sum of Squares (**SSE**) or Residual Sum of Squares is the variation that is not explained by the adopted regression model. The mean residual sum of squares is the un explained variance by the model and is distributed with $(n - 2)$ degrees of freedom.

$$SSE = \sum_{i=1}^{i=n} (y_i - \hat{y}_i)^2 , df_E = n - 2$$

The coefficient of determination (r^2), is the proportion of the variation of y-values around the mean that is predictable or explained by the x-values. It indicates how many points fall on the regression line and provides a measure of how well observed outcomes are replicated by the model (Draper, et al. 1998). The R-squared value is thus the ratio of the regression sum of squares to the total sum of squares.

$$r^2 = 1 - \frac{SSE}{SST} = \frac{SSR}{SST} = \frac{SSR}{SSYY}$$

$$r^2 = \frac{\sum_{i=1}^{i=n}(\hat{y}_i - \bar{y}_i)^2}{\sum_{i=1}^{i=n}(y_i - \bar{y}_i)^2}$$

And

$$0 \le r^2 \le 1$$

The Coefficient of Correlation r (or Multiple R in Excel) is a measure of the direction and strength of the linear association between the response signal and the concentration of the API injected.

$$r = Sign\,(\hat{a})\sqrt{r^2}, -1 \le r \le 1$$

If the adopted the model is an excellent fit, there is expected to be very little deviation between the data and the model. This implies: $SSE \to 0$ and $r^2 = 1$. The table below shows determination of R-squared values r² and r values for API alone and the API in the Dosage form sample data for this method validation.

Table 7: Determination of R-squared values r^2 and r values for API alone and the API in the Dosage form sample data for this method validation.

	API Alone (ETALON)					Dosage Form (PA+EXCIPIENTS)				
$\hat{a}$	25.0090					27.5025				
$\hat{b}$	10.6183					-135.5877				
Level	API Conc. (mg)	Observed Response Signal (mAU)	Model Predicted Signal	Explained Response Signal Variability	Total Response Signal Variability	API Conc. (mg)	Observed Response Signal (mAU	Model Predicted Signal	Explained Response Signal Variability	Total Response Signal Variability
Group	x_i	y_i	$\hat{y}_i$	$(\hat{y}_i - \bar{y}_i)^2$	$(y_i - \bar{y}_i)^2$	x_i	y_i	$\hat{y}_i$	$(\hat{y}_i - \bar{y}_i)^2$	$(y_i - \bar{y}_i)^2$
80%	72.08	1856.14	1.81E+03	2.07E+05	1.70E+05	72.08	1851.09	1.85E+03	2.50E+05	2.46E+05
	72.16	1833.31	1.82E+03	2.05E+05	1.89E+05	72.16	1878.98	1.85E+03	2.48E+05	2.19E+05
	72.40	1825.14	1.82E+03	2.00E+05	1.96E+05	72.40	1817.84	1.86E+03	2.41E+05	2.80E+05
90%	81.09	2004.49	2.04E+03	5.27E+04	6.95E+04	81.09	2060.16	2.09E+03	6.37E+04	8.23E+04
	81.18	2064.84	2.04E+03	5.16E+04	4.13E+04	81.18	2098.20	2.10E+03	6.25E+04	6.19E+04
	81.45	2053.83	2.05E+03	4.86E+04	4.59E+04	81.45	2105.88	2.10E+03	5.88E+04	5.81E+04
100%	90.10	2260.09	2.26E+03	1.74E+01	6.42E+01	90.10	2330.11	2.34E+03	2.10E+01	2.84E+02
	90.20	2241.32	2.27E+03	2.79E+00	7.17E+02	90.20	2454.19	2.35E+03	3.36E+00	1.15E+04
	90.50	2217.37	2.27E+03	3.40E+01	2.57E+03	90.50	2384.28	2.35E+03	4.12E+01	1.39E+03
110%	99.11	2507.07	2.49E+03	4.89E+04	5.71E+04	99.11	2567.26	2.59E+03	5.92E+04	4.85E+04
	99.22	2493.05	2.49E+03	5.01E+04	5.06E+04	99.22	2501.79	2.59E+03	6.06E+04	2.40E+04
	99.55	2398.53	2.50E+03	5.39E+04	1.70E+04	99.55	2572.94	2.60E+03	6.52E+04	5.11E+04
120%	108.12	2762.17	2.71E+03	1.99E+05	2.44E+05	108.12	2819.11	2.84E+03	2.41E+05	2.23E+05
	108.24	2784.32	2.72E+03	2.02E+05	2.66E+05	108.24	2859.80	2.84E+03	2.44E+05	2.63E+05
	108.60	2719.79	2.73E+03	2.10E+05	2.04E+05	108.60	2902.95	2.85E+03	2.54E+05	3.09E+05
	$\bar{x}_t$	$\bar{y}_t$		SSR	SST	$\bar{x}_t$	$\bar{y}_t$		SSR	SST
	90.27	2268.10		1.53E+06	1.55E+06	90.27	2346.97		1.85E+06	1.88E+06
r^2	0.9838					0.9842				
r	0.9919					0.9921				

From the table above, it can be noted that if the slope is not ignored, r and R-squared values approach 1, suggesting that the unrestricted model may be an excellent fit.

To see if there is a statistically significant increase in the **SSE** when the slope is ignored by the restricted model, a 5% significance level F-test is performed and both the null and alternative hypotheses specified (McCuen, R. H. 1985):

$\boldsymbol{H_0}$**:** The Restricted model: $\hat{y}_i = \bar{y}_i$ is valid and there is no increase in the residual errors
$\boldsymbol{H_1}$**:** The null hypothesis is false.

A critical F-Statistic $\left(F^{\alpha}_{q,n-k}\right)$ with a Snedecor's F-distribution with (1) degree of freedom in the numerator and $(n-2)$ degrees of freedom in the denominator at significance $\alpha = 0.05$ is compared to a Test F-Statistic (F) calculated under the assumption of the null hypothesis as the ratio of the difference between the sum of squared errors of the restricted **(SSE(R))** and unrestricted **(SSE(U))** model divided by the number of restricted parameters, in this case $q = 1$ to the sum of squared errors of the unrestricted model divided by $(n-2)$ with $k = 2$, the total number of parameters in the unrestricted model. This is a Snedecor's F distribution with (q) degrees of freedom in the

numerator and $(\boldsymbol{n-k})$ degrees of freedom in the denominator.

$$\boldsymbol{F=\frac{\frac{\chi_q^2}{q}}{\frac{\chi_{n-k}^2}{n-k}}}$$

Where $\boldsymbol{\chi_q^2}$ and $\boldsymbol{\chi_{n-k}^2}$ are Chi-Square distributions that are independent from each other.

$$\boldsymbol{F=\frac{\frac{SSE(R)-SSE(U)}{q}}{\frac{SSE(U)}{n-k}}}$$

$$\boldsymbol{F=\frac{\frac{SSE(R)-SSE(U)}{1}}{\frac{SSE(U)}{n-2}}}$$

Using the R-squared formula:

$$\boldsymbol{F=\frac{\frac{R^2(U)-R^2(R)}{q}}{\frac{1-R^2(U)}{n-k}}}$$

The null hypothesis is rejected if $\boldsymbol{F_{q,n-k}^{\alpha} \leq F}$.

The tables below show the determination of the residual sum of squares for the Unrestricted and Restricted models for Ondansetron hydrochloride dihydrate alone and in the syrup and the Analysis of Variance (ANOVA).

Table 8: Determination of the residual sum of squares for the Unrestricted model for Ondansetron hydrochloride dihydrate alone and in the syrup.

API Alone (ETALON)						Dosage Form (PA+EXCIPIENTS)					
$\hat{a}$	25.0090					$\hat{a}$	27.5025				
$\hat{b}$	10.6183					$\hat{b}$	-135.5877				
API Conc. (mg)	Observed Response Signal (mAU)	Model Predicted Response Signal	Unexplained Response Signal Variability	Explained Response Signal Variability	Total Response Signal Variability	API Conc. (mg)	Observed Response Signal (mAU)	Model Predicted Response Signal	Unexplained Response Signal Variability	Explained Response Signal Variability	Total Response Signal Variability
x_i	y_i	$\hat{y}_i$	$(y_i-\hat{y}_i)^2$	$(\hat{y}_i-\bar{y}_i)^2$	$(y_i-\bar{y}_i)^2$	x_i	y_i	$\hat{y}_i$	$(y_i-\hat{y}_i)^2$	$(\hat{y}_i-\bar{y}_i)^2$	$(y_i-\bar{y}_i)^2$
72.08	1856.14	1.81E+03	2.13E+03	2.07E+05	1.70E+05	72.08	1851.09	1.85E+03	1.19E+00	2.50E+05	2.46E+05
72.16	1833.31	1.82E+03	1.77E+02	2.05E+05	1.89E+05	72.16	1878.98	1.85E+03	8.40E+02	2.48E+05	2.19E+05
72.40	1825.14	1.82E+03	2.64E+01	2.00E+05	1.96E+05	72.40	1817.84	1.86E+03	1.78E+03	2.41E+05	2.80E+05
81.09	2004.49	2.04E+03	1.26E+03	5.27E+04	6.95E+04	81.09	2060.16	2.09E+03	8.90E+02	6.37E+04	8.23E+04
81.18	2064.84	2.04E+03	6.17E+02	5.16E+04	4.13E+04	81.18	2098.20	2.10E+03	3.24E+00	6.25E+04	6.19E+04
81.45	2053.83	2.05E+03	1.47E+01	4.86E+04	4.59E+04	81.45	2105.88	2.10E+03	3.46E+01	5.88E+04	5.81E+04
90.10	2260.09	2.26E+03	8.10E-03	1.74E+01	6.42E+01	90.10	2330.11	2.34E+03	9.78E+01	2.10E+01	2.84E+02
90.20	2241.32	2.27E+03	8.23E+02	2.79E+00	7.17E+02	90.20	2454.19	2.35E+03	1.09E+04	3.36E+00	1.15E+04
90.50	2217.37	2.27E+03	2.77E+03	3.40E+01	2.57E+03	90.50	2384.28	2.35E+03	1.18E+03	4.12E+01	1.39E+03
99.11	2507.07	2.49E+03	2.91E+02	4.89E+04	5.71E+04	99.11	2567.26	2.59E+03	5.17E+02	5.92E+04	4.85E+04
99.22	2493.05	2.49E+03	9.30E+00	5.01E+04	5.06E+04	99.22	2501.79	2.59E+03	7.78E+03	6.06E+04	2.40E+04
99.55	2398.53	2.50E+03	1.03E+04	5.39E+04	1.70E+04	99.55	2572.94	2.60E+03	7.32E+02	6.52E+04	5.11E+04
108.12	2762.17	2.71E+03	2.72E+03	1.99E+05	2.44E+05	108.12	2819.11	2.84E+03	4.36E+02	2.41E+05	2.23E+05
108.24	2784.32	2.72E+03	4.14E+03	2.02E+05	2.66E+05	108.24	2859.80	2.84E+03	3.92E+02	2.44E+05	2.63E+05
108.60	2719.79	2.73E+03	1.04E+02	2.10E+05	2.04E+05	108.60	2902.95	2.85E+03	2.80E+03	2.54E+05	3.09E+05
$\bar{x}_i$	$\bar{y}_i$		SSE(U)	SSR	SST	$\bar{x}_i$	$\bar{y}_i$		SSE(U)	SSR	SST
90.27	2268.10		2.54E+04	1.53E+06	1.55E+06	90.27	2346.97		2.83E+04	1.85E+06	1.88E+06

Table 9: Determination of the residual sum of squares for the Restricted model for Ondansetron hydrochloride dihydrate alone and in the syrup.

API Alone (ETALON)						Dosage Form (PA+EXCIPIENTS)					
$\hat{a}$	25.0090					$\hat{a}$	27.5025				
$\hat{b}$	10.6183					$\hat{b}$	-135.5877				
API Conc. (mg)	Observed Response Signal (mAU)	Model Predicted Response Signal	Unexplained Response Signal Variability	Explained Response Signal Variability	Total Response Signal Variability	API Conc. (mg)	Observed Response Signal (mAU)	Model Predicted Response Signal	Unexplained Response Signal Variability	Explained Response Signal Variability	Total Response Signal Variability
x_i	y_i	$\hat{y}_i$	$(y_i-\hat{y}_i)^2$	$(\hat{y}_i-\bar{y}_i)^2$	$(y_i-\bar{y}_i)^2$	x_i	y_i	$\hat{y}_i$	$(y_i-\hat{y}_i)^2$	$(\hat{y}_i-\bar{y}_i)^2$	$(y_i-\bar{y}_i)^2$
72.08	1856.14	2.27E+03	1.70E+05	0.00E+00	1.70E+05	72.08	1851.09	2.35E+03	2.46E+05	0.00E+00	2.46E+05
72.16	1833.31	2.27E+03	1.89E+05	0.00E+00	1.89E+05	72.16	1878.98	2.35E+03	2.19E+05	0.00E+00	2.19E+05
72.40	1825.14	2.27E+03	1.96E+05	0.00E+00	1.96E+05	72.40	1817.84	2.35E+03	2.80E+05	0.00E+00	2.80E+05
81.09	2004.49	2.27E+03	6.95E+04	0.00E+00	6.95E+04	81.09	2060.16	2.35E+03	8.23E+04	0.00E+00	8.23E+04
81.18	2064.84	2.27E+03	4.13E+04	0.00E+00	4.13E+04	81.18	2098.20	2.35E+03	6.19E+04	0.00E+00	6.19E+04
81.45	2053.83	2.27E+03	4.59E+04	0.00E+00	4.59E+04	81.45	2105.88	2.35E+03	5.81E+04	0.00E+00	5.81E+04
90.10	2260.09	2.27E+03	6.42E+01	0.00E+00	6.42E+01	90.10	2330.11	2.35E+03	2.84E+02	0.00E+00	2.84E+02
90.20	2241.32	2.27E+03	7.17E+02	0.00E+00	7.17E+02	90.20	2454.19	2.35E+03	1.15E+04	0.00E+00	1.15E+04
90.50	2217.37	2.27E+03	2.57E+03	0.00E+00	2.57E+03	90.50	2384.28	2.35E+03	1.39E+03	0.00E+00	1.39E+03
99.11	2507.07	2.27E+03	5.71E+04	0.00E+00	5.71E+04	99.11	2567.26	2.35E+03	4.85E+04	0.00E+00	4.85E+04
99.22	2493.05	2.27E+03	5.06E+04	0.00E+00	5.06E+04	99.22	2501.79	2.35E+03	2.40E+04	0.00E+00	2.40E+04
99.55	2398.53	2.27E+03	1.70E+04	0.00E+00	1.70E+04	99.55	2572.94	2.35E+03	5.11E+04	0.00E+00	5.11E+04
108.12	2762.17	2.27E+03	2.44E+05	0.00E+00	2.44E+05	108.12	2819.11	2.35E+03	2.23E+05	0.00E+00	2.23E+05
108.24	2784.32	2.27E+03	2.66E+05	0.00E+00	2.66E+05	108.24	2859.80	2.35E+03	2.63E+05	0.00E+00	2.63E+05
108.60	2719.79	2.27E+03	2.04E+05	0.00E+00	2.04E+05	108.60	2902.95	2.35E+03	3.09E+05	0.00E+00	3.09E+05
$\bar{x}_i$	$\bar{y}_i$		SSE(R)	SSR	SST	$\bar{x}_i$	$\bar{y}_i$		SSE(R)	SSR	SST
90.27	2268.1		1.55E+06	0.00E+00	1.55E+06	90.27	2346.97		1.88E+06	0.00E+00	1.88E+06

Table 10: Analysis of Variance for the residual sum of squares of the Restricted and Unrestricted models for API alone and API dosage form sample data obtained for this method validation.

ANALYSIS OF VARIANCE (ANOVA): RESIDUAL SUMS OF SQUARES COMPARISION							
API Alone (ETALON)				Dosage Form (PA+EXCIPIENTS)			
Source of Variability	Degree of Freedom	Sum of Squares	Mean Squares (Variance)	Source of Variability	Degree of Freedom	Sum of Squares	Mean Squares (Variance)
SSE(U)	13	2.54E+04	**1.95E+03**	**SSE(U)**	13	2.83E+04	**2.18E+03**
SSE(R)	12	1.55E+06		**SSE(R)**	12	1.88E+06	
$\boldsymbol{SSE(R) - SSE(U)}$	1	1.52E+06	**1.52E+06**	$\boldsymbol{SSE(R) - SSE(U)}$	1	1.85E+06	**1.85E+06**
$\boldsymbol{F}$			**7.80E+02**	$\boldsymbol{F}$			**8.51E+02**
$\boldsymbol{F_{1,13}^{0.05}}$			**4.67E+00**	$\boldsymbol{F_{1,13}^{0.05}}$			**4.67E+00**
$\boldsymbol{H_0}$	Rejected: **$F > F_{1,13}^{0.05}$, There is increase in the residual error in the Restricted model**			$\boldsymbol{H_0}$	Rejected: **$F > F_{1,13}^{0.05}$, There is increase in the residual error in the Restricted model**		

Proof of Explained Variance by the Unrestricted Model: $\hat{y}_i = \hat{a}x_i + \hat{b}$

If the Unrestricted model is justified over the Restricted model, then the slope should be statistically significant and that this model explains most of the variance in the data but the restricted one does not. The null and alternate hypotheses are specified:

H_0: The Unrestricted model: $\hat{y}_i = \hat{a}x_i + \hat{b}$ does not explain variance in the data
H_1: The null hypothesis is false.

A critical F-Statistic $\left(F_{q,n-2}^{\alpha}\right)$ with a Snedecor's F-distribution with $(q = 1)$ degree of freedom in the numerator and $(n - 2)$ degrees of freedom in the denominator at $\alpha = 0.05$ is compared to a Test F-Statistic (F) calculated under the null hypothesis as the ratio of the variance explained by the model to the un explained variance and the null hypothesis is rejected if $F_{q,n-2}^{\alpha} \leq F$.

$$F = \frac{\frac{SSR}{df_R}}{\frac{SSE}{df_{PE}}} = \frac{\frac{SSLF}{1}}{\frac{SSPE}{n-2}}$$

Table 11: Analysis of response signal variance (ANOVA) in the Unrestricted model based on explained variance.

ANALYSIS OF VARIANCE (ANOVA): REGRESSION AND RESIDUALS							
API Alone (ETALON)				Dosage Form (PA+EXCIPIENTS)			
Source of Variability	Degree of Freedom	Sum of Squares	Mean Squares (Variance)	Source of Variability	Degree of Freedom	Sum of Squares	Mean Squares (Variance)
SSE	13	2.54E+04	**1.95E+03**	**SSE**	13	2.83E+04	**2.18E+03**
SSR	1	1.53E+06	**1.53E+06**	**SSR**	1	1.85E+06	**1.85E+06**
SST	14	1.55E+06		**SST**	14	1.88E+06	
$\boldsymbol{F}$			**7.83E+02**	$\boldsymbol{F}$			**8.50E+02**
$\boldsymbol{F_{1,13}^{0.05}}$			**4.67E+00**	$\boldsymbol{F_{1,13}^{0.05}}$			**4.67E+00**
$\boldsymbol{H_0}$	Rejected: **$F > F_{1,13}^{0.05}$, The Variance in the data is explained by the Unrestricted model**			$\boldsymbol{H_0}$	Rejected: **$F > F_{1,13}^{0.05}$, The Variance in the data is explained by the Unrestricted model**		

Test for Lack of Fit in the model: $\hat{y}_i = \hat{a}x_i + \hat{b}$

If replicate data is considered, the total sum of squared errors, **SSE** is decomposed into the Sum of Squares due to Lack of Fit (**SSLF**) and the Sum of Squares due to Pure Error (**SSPE**), the two sums of squares are computed independently of regression, that is; $\hat{y}_{ij}$=$\hat{y}_i$. The Lack of Fit Sum of Squares is the sum of squares of the difference between the predicted response signal and the local mean of all response signals corresponding to a given level. Its mean represents the signal variance that is caused by lack of fit of the regression model and is distributed with $(c - 2)$ degrees of freedom.

$$SSLF = \sum_{i=1}^{i=c} \sum_{j=1}^{j=n} \left(\hat{y}_{ij} - \overline{Y}_i\right)^2$$

$$df_{LF} = c - 2$$

The Pure Error Sum of Squares (**SSPE**) is the sum of squares of the difference between the observed response signal and the local mean (group mean) of all response signals corresponding to a given level. Its mean represents the response signal variance that is due to pure error and is distributed with $(\boldsymbol{n-c})$ degrees of freedom.

$$\boldsymbol{SSPE = \sum_{i=1}^{i=c}\sum_{j=1}^{j=n}\left(y_{ij}-\overline{Y}_i\right)^2}$$

$$\boldsymbol{df_{PE} = n-c}$$

It can thus be shown that the total sum of squares is equal to the explained sum of squares plus the unexplained sum of squares, with the total degrees of freedom following the same trend.

$$\boldsymbol{SST = SSR + SSE}$$

$$\boldsymbol{df_T = df_R + df_E = n-1}$$

The residual sum of squares is equal to the lack of fit sum of squares plus the sum of squares due to pure error

$$\boldsymbol{SSE = SSLF + SSPE}$$

$$\boldsymbol{df_E = df_{LF} + df_{PE} = n-2}$$

If the Unrestricted model is justified over the Restricted model, then the residual variance observed in the data should mainly be due to pure error and not lack of fit of the model. Both the null and alternate hypotheses for the lack of fit f-test are specified:

$\boldsymbol{H_0}$: The Unrestricted model: $\boldsymbol{\hat{y}_i = \hat{a}x_i + \hat{b}}$ fits well and there is no lack of fit.
$\boldsymbol{H_1}$: The null hypothesis is false.

A critical F-Statistic $\left(\boldsymbol{F^{\alpha}_{c-2,n-c}}\right)$ with a Snedecor's F-distribution with $(\boldsymbol{c-2})$ degrees of freedom in the numerator and $(\boldsymbol{n-c})$ degrees of freedom in the denominator at $\alpha = 0.05$ is compared to a Test F-Statistic $(\boldsymbol{F})$ calculated under the null hypothesis as the ratio of the variance due to lack of fit of the Unrestricted model to the variance due to pure error and the null hypothesis is rejected if $\boldsymbol{F^{\alpha}_{c-2,n-c} \leq F}$.

$$\boldsymbol{F = \frac{\frac{SSLF}{df_{LF}}}{\frac{SSPE}{df_{PE}}} = \frac{\frac{SSLF}{c-2}}{\frac{SSPE}{n-c}}}$$

The tables below show determination of Lack of fit sum of squares and pure error sum of squares using the Unrestricted model and their ANOVA.

Table 12: Determination of Lack of fit sum of squares and pure error sum of squares using the Unrestricted model.

API Alone (ETALON)						Dosage Form (PA+EXCIPIENTS)					
$\hat{a}$	25.0090					$\hat{a}$	27.5025				
$\hat{b}$	10.6183					$\hat{b}$	-135.5877				
API Level (%)	Observed Response Signal (mAU)	API Level Response Signal Mean	Model Predicted Response Signal	Response Signal Variability due to Lack of Fit	Response Signal Variability due to Pure Error	API Level (%)	Observed Response Signal (mAU)	API Level Response Signal Mean	Model Predicted Response Signal	Response Signal Variability due to Lack of Fit	Response Signal Variability due to Pure Error
l_i	y_{ij}	$\overline{Y}_i$	$\hat{y}_{ij}$	$(\hat{y}_{ij}-\overline{Y}_i)^2$	$(y_{ij}-\overline{Y}_i)^2$	l_i	y_{ij}	$\overline{Y}_i$	$\hat{y}_{ij}$	$(\hat{y}_{ij}-\overline{Y}_i)^2$	$(y_{ij}-\overline{Y}_i)^2$
80	1856.14	1838.20	1.81E+03	7.95E+02	3.22E+02	80	1851.09	1849.30	1.85E+03	4.90E-01	3.20E+00
	1833.31		1.82E+03	3.31E+02	2.39E+01		1878.98		1.85E+03	4.90E-01	8.81E+02
	1825.14		1.82E+03	3.31E+02	1.71E+02		1817.84		1.86E+03	1.14E+02	9.90E+02
90	2004.49	2041.05	2.04E+03	1.10E+00	1.34E+03	90	2060.16	2088.08	2.09E+03	3.69E+00	7.80E+02
	2064.84		2.04E+03	1.10E+00	5.66E+02		2098.20		2.10E+03	1.42E+02	1.02E+02
	2053.83		2.05E+03	8.01E+01	1.63E+02		2105.88		2.10E+03	1.42E+02	3.17E+02
100	2260.09	2239.59	2.26E+03	4.17E+02	4.20E+02	100	2330.11	2389.53	2.34E+03	2.45E+03	3.53E+03
	2241.32		2.27E+03	9.25E+02	2.99E+00		2454.19		2.35E+03	1.56E+03	4.18E+03
	2217.37		2.27E+03	9.25E+02	4.94E+02		2384.28		2.35E+03	1.56E+03	2.76E+01
110	2507.07	2466.22	2.49E+03	5.65E+02	1.67E+03	110	2567.26	2547.33	2.59E+03	1.82E+03	3.97E+02
	2493.05		2.49E+03	5.65E+02	7.20E+02		2501.79		2.59E+03	1.82E+03	2.07E+03
	2398.53		2.50E+03	1.14E+03	4.58E+03		2572.94		2.60E+03	2.77E+03	6.56E+02
120	2762.17	2755.43	2.71E+03	2.06E+03	4.54E+01	120	2819.11	2860.62	2.84E+03	4.25E+02	1.72E+03
	2784.32		2.72E+03	1.26E+03	8.35E+02		2859.80		2.84E+03	4.25E+02	6.72E-01
	2719.79		2.73E+03	6.47E+02	1.27E+03		2902.95		2.85E+03	1.13E+02	1.79E+03
				SSLF	SSPE					SSLF	SSPE
				1.00E+04	1.26E+04					1.34E+04	1.75E+04

Table 13: Lack of fit test of the Unrestricted model (ANOVA).

ANALYSIS OF VARIANCE (ANOVA): LACK OF FIT AND PURE ERROR							
API Alone (ETALON)				**Dosage Form (PA+EXCIPIENTS)**			
Source of Variability	Degree of Freedom	Sum of Squares	Mean Squares (Variance)	Source of Variability	Degree of Freedom	Sum of Squares	Mean Squares (Variance)
SSE	13	2.54E+04		**SSE**	13	2.83E+04	
SSLF	3	**1.00E+04**	3.33E+03	**SSLF**	3	**1.34E+04**	4.47E+03
SSPE	10	**1.26E+04**	1.26E+03	**SSPE**	10	**1.75E+04**	1.75E+03
SSR	1	1.53E+06		**SSR**	1	1.85E+06	
SST	14	1.55E+06		**SST**	14	1.88E+06	
F			**2.65E+00**	F			**2.55E+00**
$F_{3,10}^{0.05}$			**3.71E+00**	$F_{3,10}^{0.05}$			**3.71E+00**
H_0	Not Rejected: $F < F_{3,10}^{0.05}$, **The Unrestricted model fits well and there is no lack of fit**			H_0	Not Rejected: $F < F_{3,10}^{0.05}$, **The Unrestricted model fits well and there is no lack of fit**		

Proof that The Unrestricted Model Passes Through the Origin: $\hat{y}_i = \hat{a}x_i$

If the adopted model describes a perfect linear relationship, it is assumed that this line should pass through the origin with a very statistically significant slope and a statistically negligible intercept. The model should thus be of the form: $\hat{y}_i = ax_i$. Under the assumptions of the Classical Linear Model, the sample regression slope and intercept follow a normal distribution with mean equal to the true population regression slope a and intercept b respectively and variance equal to the variance of the sample regression slope and intercept respectively (Uriel, E. 2013).

$$\hat{a} \sim N\left(a, \sqrt{Var(\hat{a})}\right)$$

And

$$\hat{b} \sim N\left(b, \sqrt{Var(\hat{b})}\right)$$

On the basis of the Central Limit Theorem, if the number of observations is greater than 30, it follows that the z-score or standard score, which is the number of standard deviations each sample regression estimate is from its true population regression value is a random variable which is normally distributed with mean 0 and standard deviation 1.

For $\boldsymbol{n > 30}$;

$$\frac{\hat{a} - a}{\sqrt{Var(\hat{a})}} \sim N(0, 1)$$

And

$$\frac{\hat{b} - b}{\sqrt{Var(\hat{b})}} \sim N(0, 1).$$

However, if the number of observations is equal to or less than 30, the standardised score follows a *t*-distribution with $(n - k)$ degrees of freedom. The *t*-distribution takes into account that the true population regression variance (σ^2) is unknown. This variance is estimated with an estimator $(\hat{\sigma}^2)$ and thus the uncertainty of not knowing it increases with decreasing degrees of freedom.

For $\boldsymbol{n \leq 30}$;

$$\frac{\hat{a} - a}{\sqrt{Var(\hat{a})}} \sim t_{n-k}$$

And

$$\frac{\hat{b} - b}{\sqrt{Var(\hat{b})}} \sim t_{n-k}$$

Determination of the Residual Standard Deviation (S_{yx}), the Standard Error of the Slope Estimate $(\hat{S}_a)$ and the Standard Error of the Intercept Estimate $(\hat{S}_b)$

The Residual Standard Deviation (S_{yx}) is the standard deviation of points around a regression function (Morrison, F. A. 2014). It is also referred to as the Standard Error of the Estimate or Standard Error of Mean. It is not the same as standard error in descriptive statistics! It is the estimated standard deviation of the response signal and is equal to the square root of the mean sum of squared residuals of the response signal based on the proposed model.

The Standard Error of the sample regression Slope $(\hat{S}_a)$ is the ratio of Residual Standard Deviation to the total sum $(SSxx)$ of the squared differences

between each API concentration and the mean API concentration injected.

$$S_{yx} = \sqrt{\frac{SSE}{df_E}}$$

$$S_{yx} = \sqrt{\frac{SSE}{n-2}}$$

$$S_{yx} = \sqrt{\frac{\sum_{i=1}^{i=n}(y_i - \hat{y}_i)^2}{n-2}}$$

The total sum of variations ($SSxx$) in the injected API concentration is given by:

$$SSxx = \sum_{i=1}^{i=n}(x_i - \bar{x}_\iota)^2$$

The Standard Error of the sample regression Slope ($\hat{S}_a$) is thus given by:

$$\hat{S}_a = \frac{S_{yx}}{SSxx}$$

$$\hat{S}_a = \frac{\sqrt{\frac{\sum_{i=1}^{i=n}(y_i - \hat{y}_i)^2}{n-2}}}{\sum_{i=1}^{i=n}(x_i - \bar{x}_\iota)^2}$$

The Standard Error of the sample regression Intercept ($\hat{S}_b$) can be determined using the formula:

$$\hat{S}_b = \sqrt{S_{yx}^2\left(\frac{1}{n} + \frac{\bar{x}_\iota^{\,2}}{SSxx}\right)}$$

The table below shows determination of Residual Standard Deviation and the standard error of the sample regression slope and intercept for the API alone and the API in the Dosage form.

Table 14: Determination of Residual Standard Deviation and the standard error of the sample regression slope and intercept for the API alone and the API in the Dosage form.

	API Alone (ETALON)					Dosage Form (PA+EXCIPIENTS)				
$\hat{a}$	25.0090					27.5025				
$\hat{b}$	10.6183					-135.5877				
Level	API Conc. (mg)	Observed Response Signal (mAU)	Model Predicted Response Signal	Unexplained Response Signal Variability	API Concentration Variability	API Conc. (mg)	Observed Response Signal (mAU)	Model Predicted Response Signal	Unexplained Response Signal Variability	API Concentration Variability
Group	x_i	y_i	$\hat{y}_i$	$(y_i - \hat{y}_i)^2$	$(x_i - \bar{x}_\iota)^2$	x_i	y_i	$\hat{y}_i$	$(y_i - \hat{y}_i)^2$	$(x_i - \bar{x}_\iota)^2$
80%	72.08	1856.14	1.81E+03	2.13E+03	3.30E+02	72.08	1851.09	1.85E+03	1.19E+00	3.31E+02
	72.16	1833.31	1.82E+03	1.77E+02	3.27E+02	72.16	1878.98	1.85E+03	8.40E+02	3.28E+02
	72.40	1825.14	1.82E+03	2.64E+01	3.19E+02	72.40	1817.84	1.86E+03	1.78E+03	3.19E+02
90%	81.09	2004.49	2.04E+03	1.26E+03	8.41E+01	81.09	2060.16	2.09E+03	8.90E+02	8.42E+01
	81.18	2064.84	2.04E+03	6.17E+02	8.23E+01	81.18	2098.20	2.10E+03	3.24E+00	8.26E+01
	81.45	2053.83	2.05E+03	1.47E+01	7.69E+01	81.45	2105.88	2.10E+03	3.46E+01	7.77E+01
100%	90.10	2260.09	2.26E+03	8.10E-03	2.89E-02	90.10	2330.11	2.34E+03	9.78E+01	2.78E-02
	90.20	2241.32	2.27E+03	8.23E+02	4.90E-03	90.20	2454.19	2.35E+03	1.09E+04	4.44E-03
	90.50	2217.37	2.27E+03	2.77E+03	5.29E-02	90.50	2384.28	2.35E+03	1.18E+03	5.44E-02
110%	99.11	2507.07	2.49E+03	2.91E+02	7.80E+01	99.11	2567.26	2.59E+03	5.17E+02	7.82E+01
	99.22	2493.05	2.49E+03	9.30E+00	7.97E+01	99.22	2501.79	2.59E+03	7.78E+03	8.02E+01
	99.55	2398.53	2.50E+03	1.03E+04	8.70E+01	99.55	2572.94	2.60E+03	7.32E+02	8.62E+01
120%	108.12	2762.17	2.71E+03	2.72E+03	3.18E+02	108.12	2819.11	2.84E+03	4.36E+02	3.19E+02
	108.24	2784.32	2.72E+03	4.14E+03	3.21E+02	108.24	2859.80	2.84E+03	3.92E+02	3.23E+02
	108.60	2719.79	2.73E+03	1.04E+02	3.36E+02	108.60	2902.95	2.85E+03	2.80E+03	3.36E+02
	$\bar{x}_\iota$	$\bar{y}_\iota$		SSE	SSxx	$\bar{x}_\iota$	$\bar{y}_\iota$		SSE	SSxx
	90.27	2268.10		2.54E+04	2.44E+03	90.27	2346.97		2.83E+04	2.44E+03
S_{YX}	44.0556					47.7593				
$\hat{S}_a$	0.8910					0.9659				
$\hat{S}_b$	81.2273					88.0559				

95% Confidence Interval of the slope and intercept

To test for the statistical significance of the slope, an $\alpha = 0.05$ confidence level hypothesis test is conducted according to standard hypothesis testing procedures using the Student's t-test. Both the null and alternative hypotheses are stated:

$\boldsymbol{H_0}$: The gradient is statistically insignificant: $|\hat{a} - 0| = 0 \Longrightarrow \hat{a} = 0$

$\boldsymbol{H_1}$: The null hypothesis is false: $|\hat{a} - 0| > 0 \Longrightarrow \hat{a} \neq 0$.

A critical t-statistic $\left(t_{\alpha/2,n-2}^{\alpha}\right)$ with a Student's *t*-distribution with $(n-2)$ degrees of freedom at significance level $\alpha = 0.05$ is compared to a test t-statistic (t) calculated under the null hypothesis and the null hypothesis rejected if:

$$t_{\alpha/2,n-2}^{\alpha} \leq t \Longrightarrow \rho - value < \alpha$$

$$t = \frac{|\hat{a} - 0|}{\hat{S}_a} = \frac{|\hat{a}|}{\hat{S}_a}$$

A 95 % confidence interval that is likely to contain the true unknown value of the population regression

slope is constructed under the assumption of the null hypothesis with a 2-tail *t*-distribution with $(n-2)$ degrees of freedom.

Since the gradient represents how much the HPLC detector responds to changes in the concentration, the constructed 95% confidence interval is examined to see whether it excludes 0. If this interval does not contain 0, it means that there is a 95% chance that the true gradient is not 0. The likelihood that the slope is 0 is thus ruled out. From this statistic, it is concluded that there is a linear correlation between the API concentration and the detector signal (Montgomery, & Runger, 2010).

The 95% t-Confidence Interval is:

$$\boldsymbol{CI_{0.95} = \hat{a} \pm \hat{S}_a * t^{\alpha}_{\alpha/2,n-2}}$$

Or

$$\boldsymbol{CI_{0.95} = -\hat{S}_a * t^{\alpha}_{\alpha/2,n-2} \leq a \leq \hat{S}_a * t^{\alpha}_{\alpha/2,n-2}}$$

Or

$$\boldsymbol{CI_{0.95} = \left[-\hat{S}_a * t^{\alpha}_{\alpha/2,n-2}, \hat{S}_a * t^{\alpha}_{\alpha/2,n-2}\right]}$$

To test for the statistical significance of the intercept, an $\alpha = 0.05$ confidence level hypothesis test is conducted on the intercept following the same steps of the slope test and both the null and alternative hypotheses specified and the statistics determined.

$\boldsymbol{H_0}$: The intercept is not statistically significant: $\boldsymbol{\left|\hat{b} - 0\right| = 0 \Longrightarrow \hat{b} = 0}$

$\boldsymbol{H_1}$: The null hypothesis is false: $\boldsymbol{\left|\hat{b} - 0\right| > 0 \Longrightarrow \hat{b} \neq 0}$

A critical t-statistic $\left(\boldsymbol{t^{\alpha}_{\alpha/2,n-2}}\right)$ with a Student's t-distribution with $(n-2)$ degrees of freedom at significance level $\alpha = 0.05$ is compared to a test statistic $(\boldsymbol{t})$ calculated assuming the null hypothesis is true and the null hypothesis rejected if: $\boldsymbol{t^{\alpha}_{\alpha/2,n-2} \leq t \Longrightarrow \rho - value < \alpha}$

$$\boldsymbol{t = \frac{\left|\hat{b} - 0\right|}{\hat{S}_b} = \frac{\left|\hat{b}\right|}{\hat{S}_b}}$$

The 95% t-Confidence Interval is:

$$\boldsymbol{CI_{0.95} = \hat{b} \pm \hat{S}_b * t^{\alpha}_{\alpha/2,n-2}}$$

Or

$$\boldsymbol{CI_{0.95} = -\hat{S}_b * t^{\alpha}_{\alpha/2,n-2} \leq b \leq \hat{S}_b * t^{\alpha}_{\alpha/2,n-2}}$$

Or

$$\boldsymbol{CI_{0.95} = \left[-\hat{S}_b * t^{\alpha}_{\alpha/2,n-2}, \hat{S}_b * t^{\alpha}_{\alpha/2,n-2}\right]}$$

The intercept represents the mean of HPLC detector response signal at 0 API concentration. A 95% confidence interval that includes zero means that there is a 95% chance that the intercept is actually 0, indicating it is not statistically significant. The figure below shows rejection region using a t-distribution: 2-tail alternative hypothesis (on the left) and p-value using t-distribution: 2-tail alternative hypothesis (on the right).

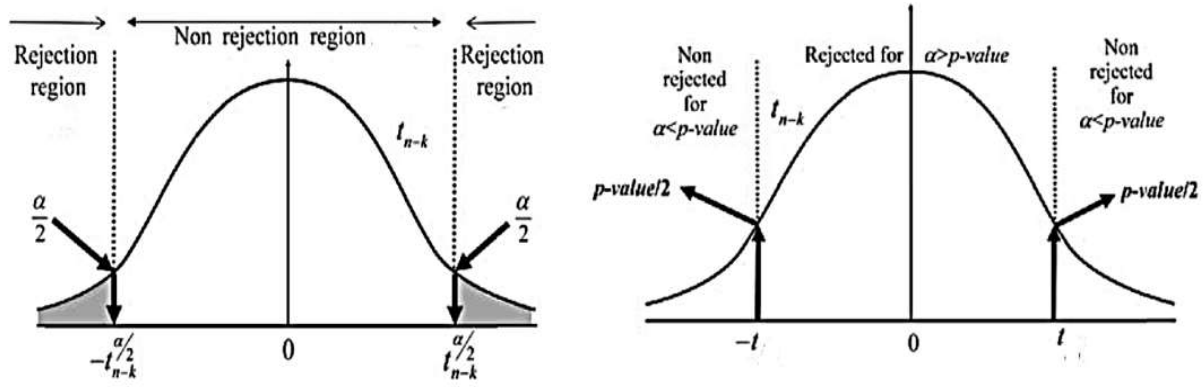


Figure 4: Rejection region using t: 2-tail alternative hypothesis (on the left) and p-value using t: 2-tail alternative hypothesis (on the right)

The table below shows Student's t-test results, 95% confidence intervals and statistical significances of the slope and intercept. From this table, we note that the model has a significant slope and an insignificant intercept 95% of the time.

To conclude, Ondansetron hydrochloride dihydrate has a coefficient of determination of 0.9842 and a coefficient of correlation of 0.9921 in the reconstituted syrup (dosage form) as indicated by the regression of the validation standards, and a coefficient of determination of 0.9838 and a coefficient of correlation of 0.9919 in samples containing no matrix (API alone) as indicated by the calibration standards.

All these parameters are within acceptable limits. The regression intercepts in both cases are not significantly far from zero. It can therefore be concluded that there is a linear correlation between the concentration of Ondansetron hydrochloride in injected samples and the HPLC detector response

signal with, described by a response function that passes through the origin.

Table 15: Student's t-test results, 95% confidence intervals and statistical significances of the slope and intercept.

	API Alone (ETALON)		Dosage Form (PA+EXCIPIENTS)	
$\hat{a}$	25.0090		27.5025	
$\hat{b}$	10.6183		-135.5877	
$\hat{S}_a$	0.8910		0.9659	
$\hat{S}_b$	81.2273		88.0559	
α	0.05		0.05	
$(1-\alpha)$	95%		95%	
Sample Size	15		15	
Coefficients	$\hat{a}$	$\hat{b}$	$\hat{a}$	$\hat{b}$
Test t-Statistic (t)	28.07	0.13	28.47	-1.54
Critical t-Statistic $t_{0.025,13}^{0.05}$	2.16	2.16	2.16	2.16
$\rho - value$	5.0962E-13	0.8980	4.2422E-13	0.1476
Decision on Null Hypothesis	REJECTED	NOT REJECTED	REJECTED	NOT REJECTED
Lower 95% Confidence Interval	23.0841	-164.8627	25.4158	-325.8209
Upper 95% Confidence Interval	26.9339	186.0993	29.5891	54.6455
Exclusion of Zero (0) in the Interval	EXCLUDED	INCLUDED	EXCLUDED	INCLUDED
Conclusion on Statistical Significance	SIGNIFICANT	INSIGNIFICANT	SIGNIFICANT	INSIGNIFICANT

The Study of Specificity and Selectivity

Specificity is the ability to assess unequivocally the analyte in the presence of components expected to be present. Such components may include; impurities, degradation products, and excipients. Specificity thus measures only the desired component without interference from other species. Selectivity is the ability of the analytical method to resolve each and every related compound in the mixture. Specificity is required for assay but selectivity is not. Both specificity and selectivity are required for impurities analysis (CDER, 1994).

The analyte should have no interference from other extraneous components and should be well resolved from them. A representative HPL chromatogram or profile should be generated and submitted to show that the extraneous peaks either by addition of known compounds or samples from stress testing are baseline resolved from the parent analyte. Examples of the extraneous peaks are as follows: For the drug substance or raw material, the related substances to consider are process impurities (which include isomeric impurities) from the synthesis process, residual pesticides, solvents, and other extraneous components from extracts of natural origin. For the drug product, the related substances may be impurities present in the active drug, degradation products, interaction of the active drug with excipients, extraneous components, e.g., residual solvents from the excipients or manufacturing process, leachable or extractables from the container and closure system or from the manufacturing process (CDER, 1994).

Specificity and selectivity are determined by analysing blanks, sample matrix (placebo), and known related impurities to determine whether interferences occur. Specificity and selectivity are also demonstrated during forced degradation studies. The chromatograms presented should be legible, labelled, and the time or time scale and attenuation should be indicated (CDER, 1994).

Peak Interference Test

To check if there is interference by the placebo, 50µl of the **PLACEBO A 100%** are injected and the chromatogram obtained is compared to two chromatograms; one obtained by injecting 50µl of the sample solution of **ETALON A 100%** and the other obtained by injecting 50µl of the **PA+EXCIPIENTS A100%** and inspected for presence of peaks at the retention time of Ondansetron hydrochloride dihydrate (Boudis H, 2015).

The three chromatograms below obtained for this method validation indicate no interference at the retention time of interest and thus excipients do not elute in the elution zone of the active ingredient.

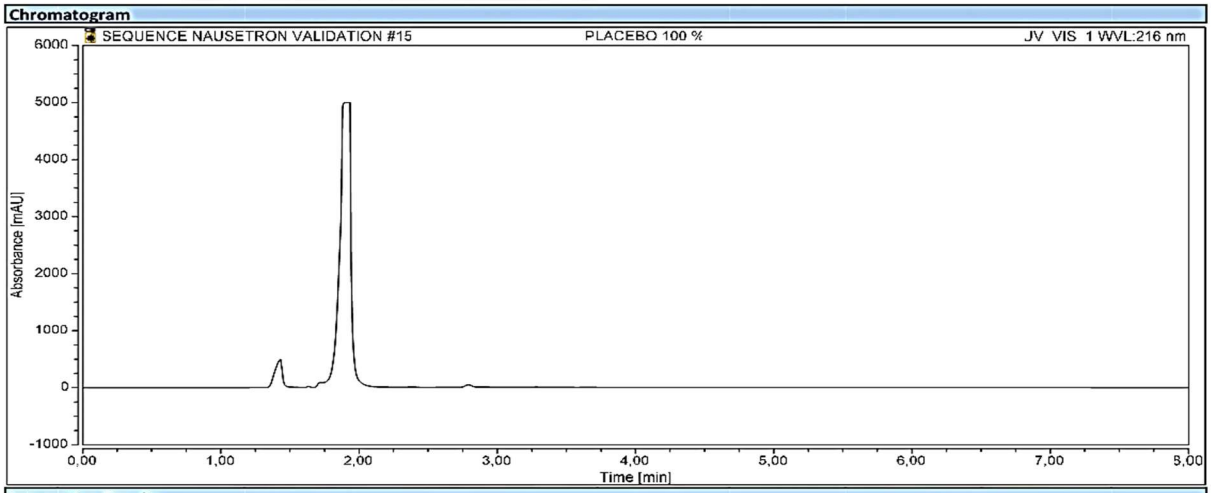
Instrument:U3000 Sequence:SEQUENCE NAUSETRON VALIDATION Page 1 of 1

Chromatogram and Results

Injection Details

Injection Name:	**PLACEBO 100 %**	*Run Time (min):*	**8,00**
Vial Number:	**BB5**	*Injection Volume:*	**50,00**
Injection Type:	**Calibration Standard**	*Channel:*	**UV_VIS_1**
Calibration Level:		*Wavelength:*	**216,0**
Instrument Method:	**INSRUMENTNAUSETRON VALIDATION**	*Bandwidth:*	**n.a.**
Processing Method:	**New ProcMethod**	*Dilution Factor:*	**1,0000**
Injection Date/Time:	**04/févr./16 12:58**	*Sample Weight:*	**1,0000**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
Total:			**0,000**	**0,000**	**0,00**	**0,00**	

Figure 5: Chromatogram obtained by injecting the sample matrix (placebo).

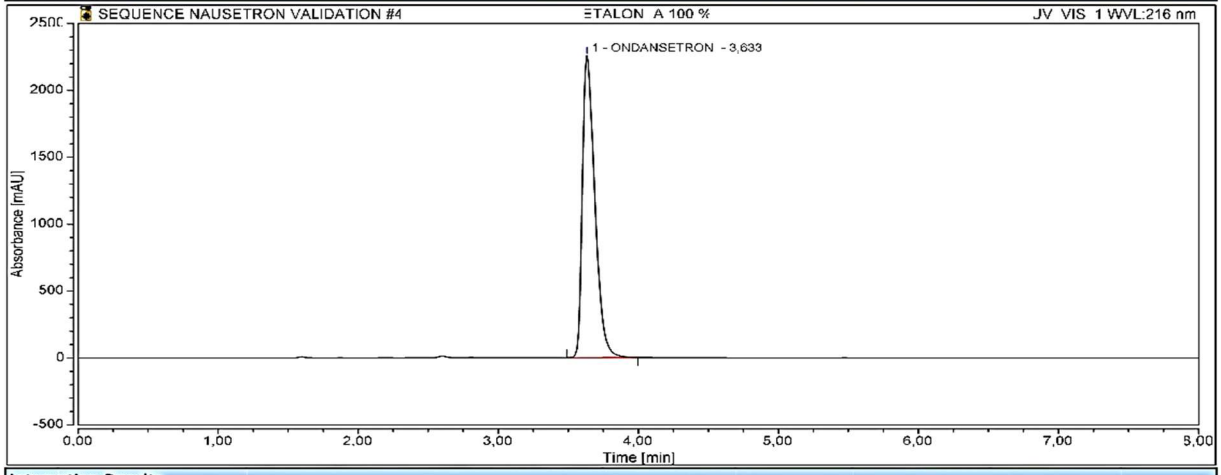
Instrument:U3000 Sequence:SEQUENCE NAUSETRON VALIDATION Page 1 of 1

Chromatogram and Results

Injection Details

Injection Name:	**ETALON A 100 %**	*Run Time (min):*	**8,00**
Vial Number:	**BA4**	*Injection Volume:*	**50,00**
Injection Type:	**Calibration Standard**	*Channel:*	**UV_VIS_1**
Calibration Level:		*Wavelength:*	**216,0**
Instrument Method:	**INSRUMENTNAUSETRON VALIDATION**	*Bandwidth:*	**n.a.**
Processing Method:	**New ProcMethod**	*Dilution Factor:*	**1,0000**
Injection Date/Time:	**04/févr./16 11:01**	*Sample Weight:*	**1,0000**

Chromatogram



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount
1	ONDANSETRON	3,633	233,011	2260,089	100,00	100,00	n.a.
Total:			**233,011**	**2260,089**	**100,00**	**100,00**	

Figure 6: Chromatogram obtained by injecting a solution of API alone at 100%.

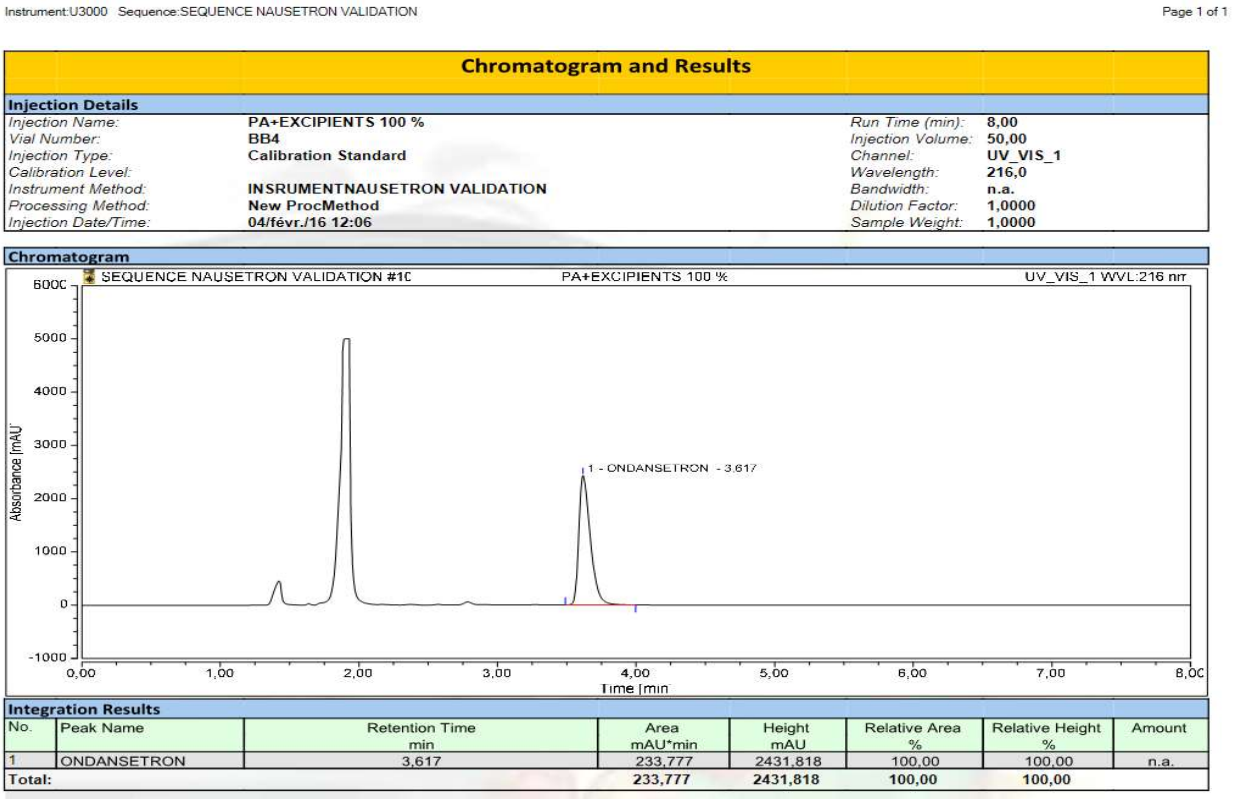


Figure 7: Chromatogram obtained by injecting a spiked placebo at 100% Ondansetron hydrochloride dihydrate.

The Matrix Effect and Systematic Error

Test for the Matrix Effect and Matrix Interference

A matrix effect in analytical chemistry refers to the combined effect of all components of the sample other than the analyte on the measurement of the quantity. If a specific component can be identified as causing an effect then this is referred to as interference (McNaught, et al, 1997).

According to the SFSTP guidelines, the matrix effect can be assessed using the Student's *t*-test by testing if there is a statistically significant difference between the slope for the calibration standard of the analyte and the slope for the validation standard of the analyte (Boudis H, 2015). Existence of this difference suggests presence of the matrix effect which thus requires the method to be reviewed. The null and alternate hypotheses are specified:

$\boldsymbol{H_0}$: There is no difference between the regression slopes.
$\boldsymbol{H_1}$: The null hypothesis is false.

The table below shows the assessment of the matrix effect using the Student's t-test on the gradients.

Table 16: Student's t-test for comparison of slopes (assessment of the matrix effect).

	API Alone (ETALON)	**Dosage Form (PA+EXCIPIENTS)**
$\widehat{a}$	**25.0090**	**27.5025**
$\widehat{b}$	**10.6183**	**-135.5877**
$\widehat{S}_a$	**0.8910**	**0.9659**
$\widehat{S}_b$	**81.2273**	**88.0559**
Sample Size	**15**	**15**
	Comparison of Slopes (Test for the Matrix Effect)	
Test Statistic	$t = \frac{\lvert\widehat{a}_1 - \widehat{a}_2\rvert}{\sqrt{\widehat{S}_{\widehat{a}_1}^{\ 2} + \widehat{S}_{\widehat{a}_2}^{\ 2}}} = \mathbf{1.90}$	
Critical t-Statistic	$t^{0.05}_{0.025,26} = \mathbf{2.06}$	
$\boldsymbol{H_0}$	$t < t^{0.05}_{0.025,26}$: **Not rejected; the slopes are the same, no matrix effect, method specific**	

Test for Systematic Error

Observational error or measurement error is the difference between a measured value of a quantity and its true value (Dodge, Y. 2006). In statistics, an error is not a "mistake" and thus variability is an inherent part of the results of measurements and of the measurement process. Measurement errors are divided into two components: random errors that lead to measurable values being inconsistent when repeated measurements of a constant attribute or quantity are taken and systematic errors that are not determined by chance but are introduced by an inaccuracy involving either the observation or measurement process inherent to the system (Taylor, J. 1997). Systematic error may also refer to an error with a nonzero mean, the effect of which is not reduced when observations are averaged.

The presence of Systematic Error in a method can be assessed using the Student's *t*-test by testing if there is a statistically significant difference between the regression intercept for the API alone and the regression intercept for the API in the Dosage form. Existence of this difference suggests error due to steps of the method (not random error) and thus requires a review of the method to make corrections. Both the null and alternate hypotheses are specified:

$\boldsymbol{H_0}$: There is no difference between the regression intercepts.
$\boldsymbol{H_1}$: The null hypothesis is false:

Table 17: Student's t-test for comparison of Intercepts (assessment of systematic error).

	API Alone (ETALON)	**Dosage Form (PA+EXCIPIENTS)**
$\widehat{a}$	**25.0090**	**27.5025**
$\widehat{b}$	**10.6183**	**-135.5877**
$\widehat{S}_a$	**0.8910**	**0.9659**
$\widehat{S}_b$	**81.2273**	**88.0559**
Sample Size	**15**	**15**
	Comparison of Intercepts (Test for Systematic Error)	
Test Statistic	$t = \frac{\lvert\widehat{b}_1 - \widehat{b}_2\rvert}{\sqrt{\widehat{S}_{\widehat{b}_1}{}^2 + \widehat{S}_{\widehat{b}_2}{}^2}} = \mathbf{1.22}$	
Critical t-Statistic	$t^{0.05}_{0.025,26} = \mathbf{2.06}$	
$\boldsymbol{H_0}$	$t < t^{0.05}_{0.025,26}$: **Not rejected; the intercepts are the same, error not systematic but random (variance), method specific**	

Systematic Error Minimisation Test

To approximate the unknown concentration of the analyte from the calibration curve, the error in the unknown concentration of analyte will be minimal if the response signal from this analyte concentration lies in the middle of the signals of all the standards. By comparing the regression intercept to zero using the Student's *t*-test, a statistically significant difference is proof that the calibration curve does not minimise the error in the unknown concentration of the analyte and the method is not specific. Both the null and alternative hypotheses are specified:

$\boldsymbol{H_0}$: There is no difference between the intercept and zero: $\lvert\widehat{b} - 0\rvert = 0 \Longrightarrow \widehat{b} = 0$, method is specific.
$\boldsymbol{H_1}$: The null hypothesis is false: $\lvert\widehat{b} - 0\rvert > 0 \Longrightarrow \widehat{b} \neq 0$.

Table 18: Test for Minimisation of Error in the Unknown API Concentration (Student's t-test for Comparison of intercept with zero)

	API Alone (ETALON)	**Dosage Form (PA+EXCIPIENTS)**
$\widehat{a}$	**25.0090**	**27.5025**
$\widehat{b}$	**10.6183**	**-135.5877**
$\widehat{S}_a$	**0.8910**	**0.9659**
$\widehat{S}_b$	**81.2273**	**88.0559**
Sample Size	**15**	**15**
	Comparing the Intercept to Zero	Comparing the Intercept to Zero
Test Statistic	$t = \frac{\lvert\widehat{b} - 0\rvert}{\widehat{S}_b} = \frac{\lvert\widehat{b}\rvert}{\widehat{S}_b} = \mathbf{0.13}$	$t = \frac{\lvert\widehat{b} - 0\rvert}{\widehat{S}_b} = \frac{\lvert\widehat{b}\rvert}{\widehat{S}_b} = \mathbf{1.54}$
Critical t-Statistic	$t^{0.05}_{0.025,13} = \mathbf{2.16}$	$t^{0.05}_{0.025,13} = \mathbf{2.16}$
$\boldsymbol{H_0}$	$t < t^{0.05}_{0.025,13}$: **Not rejected; method specific.**	$t < t^{0.05}_{0.025,13}$: **Not rejected; method specific.**

The study of specificity and selectivity confirms that no peak interference was observed at the retention time of Ondansetron hydrochloride dihydrate. No statistically significant difference was found between the regression gradient of the calibration standards and that of the validation standards. Regression intercepts in both cases are found to be not significantly different from each other as well, suggesting no matrix interference. Both regression intercepts are not significantly different from zero, suggesting the developed method minimises systematic error in the assays.

The Study of Trueness and Accuracy (Recovery)

Accuracy expresses the closeness of agreement between the value found and the value that is accepted as either a conventional true value or an accepted reference. It may often be expressed as the recovery by the assay of known, added amounts of analyte (Bliesner, D. M. 2006).

Recovery is defined as the proportion of the amount of analyte, present or added to the analytical portion of test material, which is extracted and presented for measurement. At present the term recovery is used in two different contexts:

i) Recovery (REC)

The yield of an analyte in a preconcentration or extraction stage in an analytical method.

ii) Apparent Recovery (AR)

To denote the ratio of the concentration found $(\boldsymbol{x_p})$, obtained from an analytical process via a calibration graph compared to the reference value $(\boldsymbol{x_i})$.

$$\boldsymbol{AR_i = \frac{x_p}{x_i} x\ 100}$$

The first use should be clearly distinguished from the second one. This is because a 100% of recovery does not necessary require a 100% yield for a separation or preconcentration stage. Hence, the IUPAC recommends using two different terms to distinguish between the two uses of recovery. The term "recovery" should be used for yield whereas the term "apparent recovery" $(\boldsymbol{AR})$ should be used to express the ratio of the concentration found versus the reference value (Bliesner, D. M. 2006).

Apparent Recoveries can be estimated from the regression function of the reconstituted dosage form using one of the two Reference Standard Systems:

i) Reference Standard System 1: **PA+EXCIPIENTS A 100%** (Validation Standard at 100%) or

ii) Reference Standard System 2: **All (PA+EXCIPIENTS) Series** (All Validation Standards)

The use of either reference system has to be justified depending on the regression model chosen and after having established its linearity (Boudis H, 2015).

Selecting a Reference Standard System

Assuming a linear relationship for all the standards, if $\boldsymbol{p}$ is the number of replicate unknowns, the predicted concentration $(\boldsymbol{x_p})$ based on a given reference standard system will have some error $\left(\widehat{\boldsymbol{S}}_{\boldsymbol{x_p}}\right)$ given by:

$$\widehat{\boldsymbol{S}}_{\boldsymbol{x_p}} = \frac{\boldsymbol{S_{yx}}}{|\widehat{\boldsymbol{a}}|} * \sqrt{\left(\frac{\boldsymbol{1}}{\boldsymbol{n}} + \frac{\boldsymbol{1}}{\boldsymbol{p}} + \frac{(\boldsymbol{y_p} - \bar{\boldsymbol{y}}_{\boldsymbol{i}})^{\boldsymbol{2}}}{\widehat{\boldsymbol{a}}^{\boldsymbol{2}}\boldsymbol{SSxx}}\right)}$$

From this equation, it can be concluded that the error in the predicted concentration will be minimal if the response signal from the unknown concentration lies in the middle of the signals of all the standards.

This concentration is therefore expected to be included in the 95% confidence interval of the API in the reconstituted dosage form sample at 100%. The reference standard system: **PA+EXCIPIENTS A 100%** falls within this interval. The Student's t-test can be used to determine the appropriate reference system by testing the null hypothesis that there is no statistically significant difference between the regression intercept of the API in the dosage form and zero (Boudis H, 2015).

$\boldsymbol{H_0}$: The intercept of dosage form API is not different from zero: $\left|\widehat{\boldsymbol{b}} - \boldsymbol{0}\right| = \boldsymbol{0} \Longrightarrow \widehat{\boldsymbol{b}} = \boldsymbol{0}$
$\boldsymbol{H_1}$: The null hypothesis is false: $\left|\widehat{\boldsymbol{b}} - \boldsymbol{0}\right| > \boldsymbol{0} \Longrightarrow \widehat{\boldsymbol{b}} \neq \boldsymbol{0}$

If the null hypothesis is rejected at $\alpha = 0.05$ and the difference is statistically significant, the reference standard system chosen is **All (PA+EXCIPIENTS) Series**. In this case, the apparent recoveries can be calculated from the equation:

$$\boldsymbol{AR_i} = \left(\frac{\boldsymbol{y_{ij}} - \widehat{\boldsymbol{b}}}{\boldsymbol{x_{ij}} * \widehat{\boldsymbol{a}}}\right) * \boldsymbol{100}$$

With;

$$\boldsymbol{x_p} = \frac{\boldsymbol{x_i . AR_i}}{\boldsymbol{100}}.$$

However, If the null hypothesis is not rejected and the difference is not statistically significant, the reference system of **PA+EXCIPIENTS A 100%** (Mean of all Validation Standards) will be used to calculate the predicted concentrations (Boudis H, 2015).

In this case, the apparent recoveries can be calculated from the equation:

$$AR_i = \left(\frac{y_{ij} * x_{100}}{x_{ij} * y_{100}}\right) * 100$$

With;

$$x_p = \frac{x_i . AR_i}{100}$$

The tables below show t-test results for the justification of using the Validation Standard at 100% (**PA+EXCIPIENTS A 100%**) as the reference standard system and the apparent recoveries of Ondansetron hydrochloride dihydrate in the reconstituted dosage form obtained using the chosen standard system.

Table 19: t-test results for the justification of using the Validation Standard at 100% as the reference standard system.

	API Alone (ETALON)	**Dosage Form (PA+EXCIPIENTS)**
$\hat{a}$	**25.0090**	**27.5025**
$\hat{b}$	**10.6183**	**-135.5877**
$\hat{S}_a$	**0.8910**	**0.9659**
$\hat{S}_b$	**81.2273**	**88.0559**
Sample Size	**15**	**15**
	Difference of Intercept from Zero	Difference of Intercept from Zero
Test Statistic	$t = \frac{\lvert\hat{b} - 0\rvert}{\hat{S}_b} = \frac{\lvert\hat{b}\rvert}{\hat{S}_b} = 0.13$	$t = \frac{\lvert\hat{b} - 0\rvert}{\hat{S}_b} = \frac{\lvert\hat{b}\rvert}{\hat{S}_b} = 1.54$
Critical t-Statistic	$t^{0.05}_{0.025,13} = 2.16$	$t^{0.05}_{0.025,13} = 2.16$
H_0	$t < t^{0.05}_{0.025,13}$: **Not rejected; Standard at 100% justified**	$t < t^{0.05}_{0.025,13}$: **Not rejected; Validation Standard at 100% justified**

Table 20: Determination of the Apparent Recoveries of API in the dosage form using the Validation Standard at 100% as the reference standard system.

		Injected API Conc. (mg)	**Observed Response Signal (mAU)**	**Predicted API Conc. (mg)**	**Apparent Recovery**	**Group Variance**
Level	**Day**	x_i	y_i	x_p	AR_i	**VAR.S**
Group 80%	**1**	72.08	1851.09	71.58	99.30	4.8524
	2	72.16	1878.98	69.06	95.70	
	3	72.40	1817.84	69.00	95.30	
Group 90%	**1**	81.09	2060.16	79.66	98.24	3.4029
	2	81.18	2098.20	77.12	94.99	
	3	81.45	2105.88	79.93	98.14	
Group 100%	**1**	90.10	2330.11	90.10	100.00	0.0000
	2	90.20	2454.19	90.20	100.00	
	3	90.50	2384.28	90.50	100.00	
Group 110%	**1**	99.11	2567.26	99.27	100.16	14.9689
	2	99.22	2501.79	91.95	92.67	
	3	99.55	2572.94	97.66	98.10	
Group 120%	**1**	108.12	2819.11	109.01	100.82	5.5309
	2	108.24	2859.80	105.11	97.11	
	3	108.60	2902.95	110.19	101.46	
		$\overline{x_i}$	$\overline{y_i}$	$\overline{x_p}$	$\overline{AR}_{GM}$	**SUM**
		90.27	**2346.97**	**88.69**	**98.13**	**28.7551**

Cochran's Test for Homogeneity of Variances (Homoscedasticity) of Apparent Recoveries

The calculated Apparent Recoveries are tested for homogeneity of variance using the Cochran's C-test (Boudis H, 2015).

This test, named after William G. Cochran, is a one-sided upper limit variance outlier test. It is used to decide if a single estimate of a variance (or a standard deviation) is significantly larger than a group of variances (or standard deviations) with which the single estimate is supposed to be comparable. The C-test has been recommended by IUPAC and ISO and should not be confused with Cochran's Q-test, which applies to the analysis of two-way randomized block designs. The C-test

assumes a balanced design, i.e. the considered full data set should consist of individual data series that all have equal size. The C-test further assumes that each individual data series is normally distributed. Although primarily an outlier test, the C-test is also in use as a simple alternative for regular homoscedasticity tests such as Bartlett's test, Levene's test and the Brown–Forsythe test to check a statistical data set for homogeneity of variances.

The C-test detects one exceptionally large variance value at a time. The corresponding data series is then omitted from the full data set. According to ISO standard 5725, the C-test may be iterated until no further exceptionally large variance values are detected, but such practice may lead to excessive rejections if the underlying data series are not normally distributed. The C-test evaluates the ratio:

$$C = \frac{S_{max}^2}{\sum_{j=1}^{j=c} S_j^2}$$

With;

$$S_j^2 = \frac{\sum_{i=1}^{i=p}(x_{ij} - \overline{x_j})^2}{p-1}$$

and

$$\overline{x_j} = \frac{\sum_{i=1}^{i=n} x_{ij}}{p}.$$

The C-test tests the null hypothesis against the alternative hypothesis:

$\boldsymbol{H_0}$: All variances are equal (homogeneous)
$\boldsymbol{H_1}$: At least one variance value is significantly larger than the other variance values.

A critical C-Statistic $\left(\boldsymbol{C_{p,c}^{\alpha}}\right)$ with $(\boldsymbol{p})$ degrees of freedom in the numerator and $(\boldsymbol{c})$ degrees of freedom in the denominator at $\alpha = 0.05$ is compared to a test C-Statistic $(\boldsymbol{C})$ calculated under the null hypothesis as the ratio of the maximum variance to the total sum of all variances from each group and the null hypothesis is rejected if $\boldsymbol{C_{p,c}^{\alpha}} \leq \boldsymbol{C}$.

The table below shows C-test results for homogeneity of variance of the apparent recoveries of Ondansetron hydrochloride dihydrate in the reconstituted syrup.

Table 21: The table below shows C-test results for homogeneity of variance of the apparent recoveries of the API in the syrup.

COCHRAN C-test for Homoscedasticity					
Groups	1	2	3	4	5
Number per group (p)	3	3	3	3	3
S^2	4.8524	3.4029	0.0000	14.9689	5.5309
s_{max}^2	**14.9689**				
$\sum_{1}^{5} S^2$	**28.7551**				
C	**0.52**				
$C_{3,5}^{0.05}$	**0.68**				
H_0	$C_{3,5}^{0.05} > C$: **Not Rejected, variances homogeneous**				

One-Way Analysis of Variance Test for Validity of Means of Apparent Recoveries

A One-Way Analysis of Variance is a way to test the equality of three or more means at one time by using variances (Boudis H, 2015). For a one-way ANOVA to be applicable, it is assumed that the populations from which the samples were obtained must be normally or approximately normally distributed; the samples must be independent and the variances of the populations must be equal (homoscedasticity should be confirmed first). Both the null and alternative hypotheses are specified:

$\boldsymbol{H_0}$: All means are equal.
$\boldsymbol{H_1}$: At least one mean is different.

A critical F-Statistic $\left(\boldsymbol{F_{c-1,n-c}^{\alpha}}\right)$ with a Snedecor's F-distribution with $(\boldsymbol{c-1})$ degrees of freedom in the numerator and $(\boldsymbol{n-c})$ degrees of freedom in the denominator at $\alpha = 0.05$ is compared to a test F-Statistic $(\boldsymbol{F})$ calculated under the null hypothesis as the ratio of the variance between the groups to the variance within the groups and the null hypothesis is rejected if $\boldsymbol{F_{c-1,n-c}^{\alpha}} \leq \boldsymbol{F}$.

The whole idea behind the One-way analysis of variance is to compare the ratio of the Between Group Variance to the Within Group Variance. If the variance caused by the interaction between the samples is much larger when compared to the variance that appears within each group, then it is because the means are not the same and the null hypothesis should be rejected.

$$F = \frac{\frac{SSB}{c-1}}{\frac{SSW}{n-c}}.$$

If we define the Grand Mean ($\bar{X}_{GM}$) of a set of samples as the total of all the data values each marked (X_i) divided by the total sample size (n):

$$\bar{X}_{GM} = \frac{\sum_{i=1}^{n} X_i}{n},$$

Then the Total Variation (**TSS**) is comprised of the sum of the squares of the differences of each data point with the grand mean and is distributed with $(c * p - 1)$ degrees of freedom.

$$TSS = \sum_{i=1}^{n} (X_i - \bar{X}_{GM})^2 , dfT = c * p - 1$$

The Between Group Variation (**SSB**) is the variation due to the interaction between the samples. If the sample means are close to each other (and therefore the Grand Mean) this will be small. There are (c) samples involved with one data value for each sample (the sample mean), so there are $(c - 1)$ degrees of freedom.

$$SSB = \sum_{i=1}^{p} p(\bar{X}_i - \bar{X}_{GM})^2 , dfB = c - 1$$

The Within Group Variation (**SSW**) is the Sum of Squares Within groups. Each sample is considered independently, no interaction between samples is involved so its distributed with $(n - c)$ degrees of freedom.

$$SSW = \sum_{i=1}^{n} (x_i - \bar{X}_i)^2 , dfW = n - c$$

The degrees of freedom are equal to the sum of the individual degrees of freedom for each sample. Since each sample has degrees of freedom equal to one less than their sample sizes, and there are (c) samples, the total degrees of freedom are (c) less than the total sample size: $dfW = n - c = c(p - 1)$. The tables below show the determination of intra and inter-group variability of the apparent recoveries of API in the dosage form and a One-way ANOVA.

Table 22: Determination of intra and inter-group variability of the apparent recoveries of API in the dosage form.

		Apparent Recovery (AR)	Group Mean AR	Group Variance	Variability from the Grand Mean	Variability Between Groups	Variability Within Groups
Level	**Day**	$\frac{x_p}{x_i} x\ 100$	$\bar{X}_i$	**VAR.S**	$\left(\left(\frac{x_p}{x_i} x\ 100\right) - \bar{X}_{GM}\right)^2$	$3(\bar{X}_i - \bar{X}_{GM})^2$	$\left(\left(\frac{x_p}{x_i} x\ 100\right) - \bar{X}_i\right)^2$
80%	**1**	99.30	96.77	4.8524	1.367	5.582	6.417
	2	95.70			5.910		1.138
	3	95.30			8.010		2.150
90%	**1**	98.24	97.12	3.4029	0.011	3.062	1.244
	2	94.99			9.857		4.534
	3	98.14			0.000		1.028
100%	**1**	100.00	100.00	0.0000	3.483	10.450	0.000
	2	100.00			3.483		0.000
	3	100.00			3.483		0.000
110%	**1**	100.16	96.98	14.9689	4.112	4.001	10.130
	2	92.67			29.826		18.545
	3	98.10			0.001		1.263
120%	**1**	100.82	99.80	5.5309	7.226	8.295	1.051
	2	97.11			1.056		7.238
	3	101.46			11.075		2.772
		$\overline{AR}_{GM}$	$\bar{X}_{GM}$	**Mean**	**TSS**	**SSB**	**SSW**
		98.13	**98.13**	**5.7510**	**88.901**	**31.390**	**57.510**

Table 23: One-Way ANOVA test performed on the apparent recoveries of API in the dosage form for the validity of means.

One-Way ANOVA Table						
	Source of variations	**Sum of Squares**	**Degrees of freedom**	**Mean Squares (Variances)**	***F***	$F_{4,10}^{0.05}$
	Total TSS	**88.9005**	14	6.35003	**1.37**	**3.48**
	Between SSB	**31.3902**	4	**7.8476**		
	Within SSW	**57.5103**	10	**5.7510**		
H_0:	$F < F_{4,10}^{0.05}$ **: Not Rejected, all the means are equal**					

Confidence Interval of the Grand Mean Apparent Recovery

If all the group variances are confirmed to be homogenous (Homoscedasticity) and all the means are confirmed to be equal (One-Way ANOVA), then a 95% confidence interval constructed for the grand mean apparent recovery should include 100% if the method is exact and accurate.

Error in the mean predicted API Concentration

The mean predicted API concentration $(\overline{x_p})$ will have some error which can be calculated from the formula; with $\boldsymbol{p}$ the number of repetitions:

$$\widehat{S}_{\overline{x_p}} = \frac{S_{yx}}{|\hat{a}|} * \sqrt{\left(\frac{1}{n} + \frac{1}{p} + \frac{(y_{\overline{x_p}} - \bar{y}_t)^2}{\hat{a}^2 SSxx}\right)}$$

In our case, the response signal $\left(y_{\overline{x_p}}\right)$ corresponding to this mean predicted API concentration can be got from:

$$\overline{AR}_{GM} = \left(\frac{y_{\overline{x_p}} * \bar{x}_{100}}{\overline{x_p} * \bar{y}_{100}}\right) * 100$$

From this;

$$98.13 = \left(\frac{y_{\overline{x_p}} * 90.27}{88.69 * 2389.53}\right) * 100$$

$$y_{\overline{x_p}} = 2303.80$$

$$\widehat{S}_{\overline{x_p}} = \frac{47.7593}{27.5025}\sqrt{\left(\frac{1}{15} + \frac{1}{3} + \frac{(2303.80 - 2346.97)^2}{27.5025^2 * 2.44 * 10^3}\right)}$$

$$\widehat{S}_{\overline{x_p}} = 1.10$$

95% Confidence Interval of the Mean Predicted API Concentration

The confidence interval of $(\overline{x_p})$ is:

$$CI_{0.95} = \overline{x_p} \pm \widehat{S}_{\overline{x_p}} * t^{\alpha}_{\alpha/2,n-2}$$

$$CI_{0.95} = 88.69 \pm 1.10 * 2.16$$

$$CI_{0.95} = [86.31 mg/l, 91.07 mg/l]$$

Thus, the confidence interval around the grand mean apparent recovery is:

$$CI_{0.95} = \left[\left(\frac{86.31}{90.27} * 100\right), \left(\frac{91.07}{90.27} * 100\right)\right]$$

$$CI_{0.95} = [95.61, 100.89]$$

The 95% confidence of the mean apparent recovery of Ondansetron hydrochloride dihydrate in samples of the reconstituted syrup (Nausetron) includes 100% and is within ±5%. Each individual sample apparent recovery lies within the range of 92% to 102%. The API in the syrup has a coefficient of determination of 0.9842 and a coefficient of correlation of 0.9921, both within acceptable limits. The y intercept of the API in the syrup is not statistically different from zero, justifying the use of the spiked placebo at 100% as reference standard.

The Study of Precision

Precision is the degree of agreement among individual test results when an analytical method is used repeatedly to multiple samplings of a homogeneous sample. The statistical study of precision is thus aimed at demonstrating the Repeatability or method precision ($\boldsymbol{re}$) and Intermediate Precision or Reproducibility ($\boldsymbol{Re}$) of the method.

Repeatability (method repeatability) or test–retest reliability is the variation in measurements taken by a single person or instrument on the same item, under the same conditions, and in a short period of time (Trochim, W. M. K. 2006). A less-than-perfect test–retest reliability causes test–retest variability. Such variability can be caused by, for example, intra-individual variability and intra-observer variability. A measurement may be said to be repeatable when this variation is smaller than a pre-determined acceptance criterion. Repeatability does not distinguish between variation from the instrument or system alone and from the sample preparation process. Repeatability is performed by analysing multiple replicates of an assay composite sample using the analytical method. The apparent recovery value is calculated and reported for each value.

Intermediate precision or method reproducibility refers to variations within a laboratory as with different days, with different instruments, by different analysts, and so forth. Intermediate precision was formally known as ruggedness. A second analyst repeats the repeatability analysis on a different day using different conditions and different instruments. The apparent recovery values are calculated and reported. A statistical comparison is made to the first analyst's results (Bliesner, D. M. 2006).

Cochran's C-test for Homoscedasticity of Apparent Recoveries from multiples injections of the Validation Standard at 100%

Three series (index j , $p = 3$) of six determinations (index i , $n = 6$) are carried out on the validation standard at 100% (**PA+EXCIPIENTS A 100%**) at a rate of one series per day. Apparent recoveries are

then calculated and tested for homogeneity of the variances using the Cochran's c-test (Boudis H, 2015). Both the null and alternative hypothesis are specified:

$\boldsymbol{H_0}$: All variances are equal.
$\boldsymbol{H_1}$: At least one variance value is significantly larger than the other variance values.

A critical C-Statistic $\left(\boldsymbol{C_{p,c}^{\alpha}}\right)$ with $(\boldsymbol{p})$ degrees of freedom in the numerator and $(\boldsymbol{c})$ degrees of freedom in the denominator at $\alpha = 0.05$ is compared to a Test C-Statistic $(\boldsymbol{C})$ calculated under the null hypothesis as the ratio of the maximum variance to the total sum of all variances from each group and the null hypothesis is rejected if $\boldsymbol{C_{p,c}^{\alpha}} \leq \boldsymbol{C}$.

The tables below show the determination of Apparent Recoveries of Validation Standard at 100% and results for Cochran's C-test for homogeneity of variance.

Table 24: Determination of Apparent Recoveries from multiples injections of the Validation Standard at 100%

Apparent Recoveries of 6 injections of the Reconstituted Syrup at 100%						
	Day 1		Day 2		Day 3	
API Injection	Response Signal	AR	Response Signal	AR	Response Signal	AR
1	2350.61	100.88	2478.24	100.98	2395.25	100.46
2	2351.78	100.93	2482.90	101.17	2400.49	100.68
3	2350.85	100.89	2479.71	101.04	2399.30	100.63
4	2353.18	100.99	2486.09	101.30	2389.76	100.23
5	2353.18	100.99	2475.79	100.88	2393.58	100.39
6	2369.72	101.70	2478.00	100.97	2393.58	100.39
Group Mean $(\bar{X}_j)$		**101.06**		**101.06**		**100.46**
Grand Mean $(\bar{X}_{GM})$	**100.86**					
Group Variance (S_j^2)		**0.1**		**0.02**		**0.03**

Table 25: Cochran's C-test results for homogeneity of variance.

COCHRAN C-test for Homoscedasticity	
S_{max}^2	**0.10**
$\sum_{1}^{3} S^2$	**0.15**
C	**0.66**
$C_{6,3}^{0.05}$	**0.68**
H_0	$C_{3,5}^{0.05} > C$: **Not Rejected, variances are the same**

Determination of Variance of Repeatability $(\boldsymbol{S_{re}^2})$***, Inter-Group Variance*** $\left(\boldsymbol{S_g^2}\right)$***, Variance of Reproducibility*** $\left(\boldsymbol{S_{Re}^2}\right)$***, Repeatability*** $(\boldsymbol{re})$***, Intermediate Precision*** $(\boldsymbol{Re})$ ***and Variation Coefficients.***

After homoscedasticity is confirmed, the Variance of Repeatability $(\boldsymbol{S_{re}^2})$ and the Inter-Group Variance $\left(\boldsymbol{S_g^2}\right)$ are determined using the 3 relationships below:

$$\boldsymbol{S_{re}^2} = \frac{\sum_{j=1}^{j=p}(\boldsymbol{n_j} - \boldsymbol{1})\boldsymbol{S_j^2}}{\left(\sum_{j=1}^{j=p}\boldsymbol{n_j}\right) - \boldsymbol{p}}$$

$$\overline{\boldsymbol{n}} = \frac{\sum_{j=1}^{j=p}\boldsymbol{n_j} - \left(\frac{\sum_{j=1}^{j=p}\boldsymbol{n_j}^2}{\sum_{j=1}^{j=p}\boldsymbol{n_j}}\right)}{\boldsymbol{p} - \boldsymbol{1}}$$

$$\text{and } \boldsymbol{S_g^2} = \frac{\left(\frac{\sum_{j=1}^{j=p}\boldsymbol{n_j}\left(\bar{\boldsymbol{X}}_{\boldsymbol{j}} - \bar{\boldsymbol{X}}_{\boldsymbol{GM}}\right)^2}{\boldsymbol{p} - \boldsymbol{1}}\right) - \boldsymbol{S_{re}^2}}{\overline{\boldsymbol{n}}}$$

If the obtained Inter-Group Variance $\left(\boldsymbol{S_g^2}\right)$ is negative, the Variance of Repeatability $(\boldsymbol{S_{re}^2})$ is assumed and taken to be equal to 0. This assumption is not problematic at all; it simply means that the variance of repeatability and Intermediate Variance $\left(\boldsymbol{S_R^2}\right)$ are substantially equal and therefore their difference is not statistically significant (Boudis H, 2015).

The Variance of Reproducibility $\left(\boldsymbol{S_{Re}^2}\right)$ is finally computed from:

$$\boldsymbol{S_R^2} = \boldsymbol{S_r^2} + \boldsymbol{S_g^2}$$

The actual values of Repeatability and Intermediate Precision or Reproducibility can be obtained from:

$$\boldsymbol{re = 2.8 * \sqrt{S_{re}^2}} \text{ and } \boldsymbol{Re = 2.8 * \sqrt{S_{Re}^2}}$$

The Coefficient of Variation of Repeatability ($\boldsymbol{CV_{re}}$) or the relative standard deviation of repeatability and the Coefficient of Variation of Reproducibility or intermediate precision ($\boldsymbol{CV_{Re}}$) are determined and a value less than 2% indicates that the method is precise (Boudis H, 2015).

$$\boldsymbol{CV_{Re} = \left(\frac{\sqrt{S_{Re}^2}}{\bar{X}_{GM}}\right) * 100}$$

and

$$\boldsymbol{CV_{re} = \left(\frac{\sqrt{S_{re}^2}}{\bar{X}_{GM}}\right) * 100}.$$

The table below shows the determination of Variance of Repeatability, Inter-Group Variance, Variance of Reproducibility, Repeatability, Intermediate Precision and their Coefficients of variation (relative standard deviation).

Table 26: Determination of Variance of Repeatability, Inter-Group Variance, Variance of Reproducibility, Repeatability, Intermediate Precision and Variation Coefficients.

	Determination of Sums			
	Day 1	**Day 2**	**Day 3**	
Number of Groups (p)	3	3	3	
	6	6	6	
Group Mean $\bar{X}_j$	101.06	101.06	100.46	
Grand Mean ($\bar{X}_{GM}$)	100.86	100.86	100.86	
Group Variance (S_j^2)	0.1	0.02	0.03	**SUM**
Repetitions par Group n_j	6	6	6	**18**
$(\bar{X}_j - \bar{X}_{GM})^2$	0.04	0.04	0.16	**0.24**
$n_j(\bar{X}_j - \bar{X}_{GM})^2$	0.24	0.24	0.96	**1.44**
$(n_j - 1)S_j^2$	0.5	0.1	0.15	**0.75**
n_j^2	36	36	36	**108**
$\bar{n}$	**6**			

Determination of Precision Coefficients				
Variability	**Variance**	**Standard Deviation**	**Value**	**Coefficient of Variation (%)**
Inter-Group	0.11	0.33	0.94	0.33
Repeatability	0.05	0.22	0.63	**0.22**
Reproducibility (Intermediate Precision)	0.16	0.40	1.13	**0.40**
Decision	**Each Coefficient of Variation is less than 2%, method is precise**			

The coefficient of variation of repeatability is of 0.22% and is within the acceptance limits of less than or equal to 2%. The coefficient of variation of intermediate precision is of 0.40% and is within the acceptance limits of less than or equal to 2%.

The Study of the Limit of detection, the Limit of Quantification and Sensitivity

The Limit of Detection (LOD) is the lowest quantity of a substance that can be distinguished from the absence of that substance (a blank value) with a stated confidence level usually 99%. It usually depends on the mean and standard deviation of the blank. It is also dependent on the accuracy of the model used to predict concentrations from the raw analytical response signal data. A number of different "detection limits" are commonly used. These include the instrument detection limit (IDL), the method detection limit (MDL), the practical quantification limit (PQL), and the limit of quantification (LOQ) (MacDougall, D., et al, 1980).

The figure below shows the relationship between the blank, the limit of detection (LOD), and the limit of quantification (LOQ) by showing the probability density function for normally distributed measurements at the blank, at the LOD defined as 3 times the standard deviation of the blank, and at the

LOQ defined as 10 times the standard deviation of the blank.

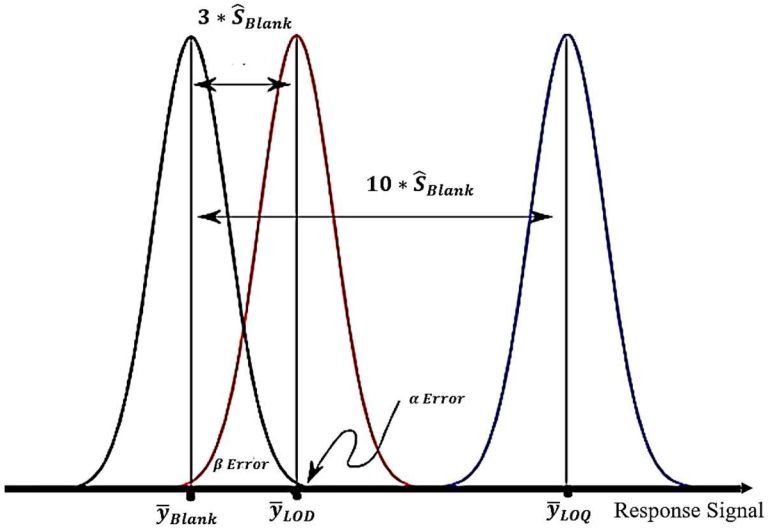


Figure 8: Illustration of the concept of detection limit and quantitation limit by showing the theoretical normal distributions associated with the blank, detection limit, and quantification limit level samples (Wikipedia)

If the null hypothesis is that the minimum analyte concentration that produces a response signal at least 3 standard deviations above the blank response signal is the limit of detection, for a response signal at the limit of detection ($\boldsymbol{y_{LOD}}$), the probability of not rejecting the null hypothesis given that it is false (alpha ($\boldsymbol{\alpha}$) error, false positive or type I error) is small at 1%. However, the probability of rejecting the null hypothesis given that it is true (beta ($\boldsymbol{\beta}$) error, false negative or type II error) is 50% for a sample that has a concentration at the LOD (red line). This means a sample could contain an impurity at the LOD, but there is a 50% chance that a measurement would give a result less than the LOD. At the LOQ (blue line), there is minimal chance of a false negative.

Instrument Detection Limit (IDL)

More often than not, analytical instruments produce a response signal even when a blank is analysed. This response signal is referred to as the noise level. The IDL is the analyte concentration that is required to produce a signal greater than three times the standard deviation of the noise level. This may be practically measured by analysing 6 or more standards at the claimed IDL or estimated IDL from preliminary studies and then calculating the standard deviation from the measured concentrations of those standards. The limit of detection is the first concentration at which the analyte has a signal-to-noise ratio of 3:1. According to IUPAC, the detection limit is the smallest concentration or absolute amount of analyte that has a signal significantly larger than the signal arising from a reagent blank (MacDougall, D., et al, 1980).

Mathematically, the analyte's signal at the detection limit ($\boldsymbol{y_{LOD}}$) is given by:

$$\boldsymbol{y_{LOD} = y_{Blank} + 3 * \left(\sqrt{Var(y_{Blank})}\right)}$$

Method Detection Limit (MDL)

In any analytical method, additional steps mean additional opportunities for error. Since detection limits are defined in terms of error, this will naturally increase the measured detection limit. A method detection limit (MDL) is therefore the limit of detection with all method steps included. A number of approaches is recommended by the ICH depending on instrument used for analysis, nature of analyte and suitability of the method. The acceptable approaches include; visual evaluation, signal-to-noise ratio, standard deviation of the response and standard deviation of the slope of linearity plot. From the linearity plot using the calibration standards, the detection limit can be approximated from:

$$\boldsymbol{LOD = \frac{3.3\hat{S}_b}{\hat{a}}}$$

A 99% confidence interval of the MDL is got by analysing 6 or more samples of concentration near the approximated or claimed method limit of detection and then finding the standard deviation of the concentrations and a t-statistic ($\boldsymbol{t_{0.01,n-1}}$) with a t-distribution with ($\boldsymbol{n-1}$) degrees of freedom at confidence level 99% obtained from a one-sided t-distribution. The interval is:

$$MDL \pm t_{0.01,n-1} * \sqrt{Var(MDL)}$$

Limit of quantitation (LOQ)

The LOQ is the limit at which the difference between two distinct values can be reasonably discerned. Since the limit of quantification may be drastically different between laboratories, the Practical Quantification Limit (PQL) is commonly used. The limit of quantitation is a parameter of quantitative assays for low levels of compounds in sample matrices and is used particularly for the determination of impurities and or degradation products or low levels of active constituent in a product (CDER, 1994).

The LOQ may be determined by preparing standard solutions at estimated LOQ concentration based on preliminary studies or from the calibration curve:

$$LOQ = \frac{10\hat{S}_b}{\hat{a}}$$

The solution should be analysed n times, normally 6 to 10 times. The average response and the standard deviation of the n results should be calculated and the standard deviation should be less than 20%. If the SD exceeds 20%, a new standard solution of higher concentration should be prepared and the above procedure repeated. The limit of quantification is the first concentration at which the analyte has a signal-to-noise ratio of 10:1.

In relation to the noise, the analyte's response signal at the limit of quantification can be obtained from:

$$y_{LOQ} = y_{Blank} + 10 * \left(\sqrt{Var(y_{Blank})}\right)$$

It should be noted that the purest analyte available should be used to determine the limit of detection and limit of quantitation (i.e., primary standard such as USP or EPCRS) although these tests are not required for Category I (assay) methods.

Sensitivity (a)

Sensitivity is the change in the response signal of a measuring instrument divided by corresponding change in the stimulus or introduced concentration (Boqué, et al, 2002). Although it clearly applies to the measuring instrument, sensitivity can also be applied to the method as a whole. If (a) and (b) represent the unobservable values of the population regression slope and intercept and $(\hat{a})$ and $(\hat{b})$ are their respective sample regression estimates obtained from the calibration standards (API alone), then sensitivity can be seen as the true population regression gradient (a). A 95% confidence interval constructed for the sample regression gradient contains the true value of sensitivity:

$$\hat{a} - \hat{S}_{\hat{a}} * t^{\alpha}_{\alpha/2,n-2} \leq a \leq \hat{a} + \hat{S}_{\hat{a}} * t^{\alpha}_{\alpha/2,n-2}$$

The table below shows the limit of detection, limit of quantification and a 95% sensitivity interval approximated from the calibration standard solutions containing Ondansetron hydrochloride dihydrate alone without excipients for this method validation.

Table 27: The limit of detection, limit of quantification and sensitivity approximated using calibration standard solutions containing the API alone.

Parameter	API alone	Units
$\hat{a}$	**25.0090**	mAUl/mg
$\hat{b}$	**10.6183**	mAU
$\hat{S}_a$	**0.8910**	mAUl/mg
$\hat{S}_b$	**81.2273**	mAU
LOD	**10.7182**	mg/l
LOQ	**32.4792**	mg/l
a	$\mathbf{23.084 \leq a \leq 26.933}$	mAUl/mg

Sensitivity is not an essential parameter during method validation, but it is very important in method optimization and quality assurance procedures for routine monitoring of the instrument's performance. Although sensitivity is not to be confused with limit of quantitation, these terms are interrelated. With a given signal to noise ratio, the higher the sensitivity, the lower the LOD and LOQ. Sensitivity is also directly related to ionization suppression in a way that the essence of ionization suppression is the decrease of sensitivity due to co-eluting compounds.

Sensitivity evaluation is of three main uses. It is used for optimization (help in maximizing sensitivity) of method parameters during method development, for daily optimization of instrument parameters and for monitoring of the instrument performance.

The Study of Range

Range is the interval between the upper and lower concentrations (amounts) of analyte in the sample (including these concentrations) for which it has

been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity. Range is normally expressed in the same units as test results (e.g., percent, parts per million) obtained by the analytical method. Range (sometimes referred to the linearity of a method) is evaluated using samples (usually spiked placebos) and must encompass the specification range of the component assayed in the drug product. The range is established by the required limits of the method and the point at which linearity is compromised (Bliesner, D. M. 2006).

Verification is made that the method provides acceptable precision, accuracy, and linearity when applied to samples at the extreme as well as within the range. For assay of drug substance or finished product, the recommended range is from 80–120% of the test concentration. For determination of an impurity, a range of 50–120% of the specification is the recommended minimum. For content uniformity, a range of 70–130% of the test concentration is recommended, unless a wider or more appropriate range is justified based upon the dosage form. For dissolution testing, ± 20% over the specified range of the dissolution test is the minimum recommended range.

The figures below show graphs of the concentration of Ondansetron hydrochloride dihydrate against the HPLC response signal in the Calibration Standard and in Nausetron Syrup and the range for which precision, accuracy and linearity was demonstrated.

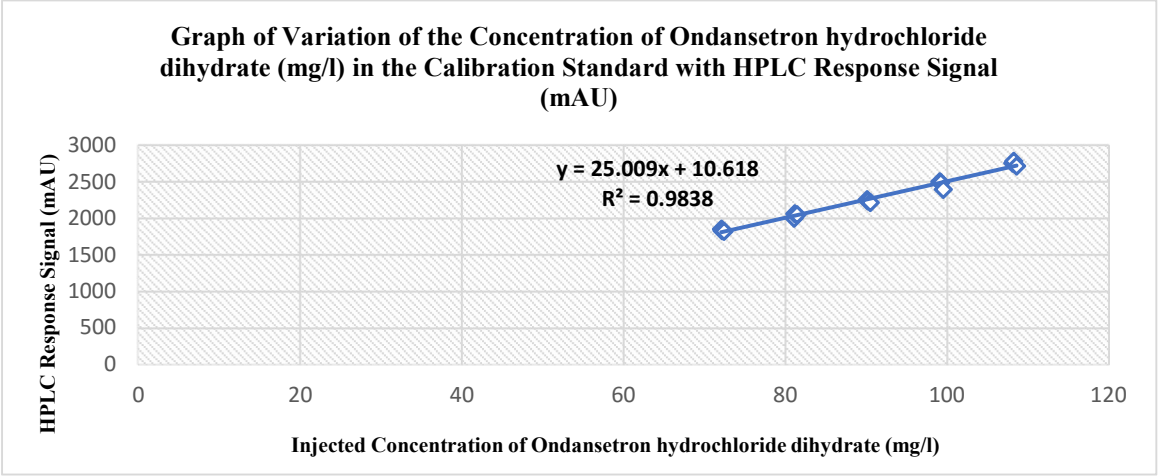


Figure 9: Graph of concentration of Ondansetron hydrochloride dihydrate against the HPLC response signal in the Calibration Standard.

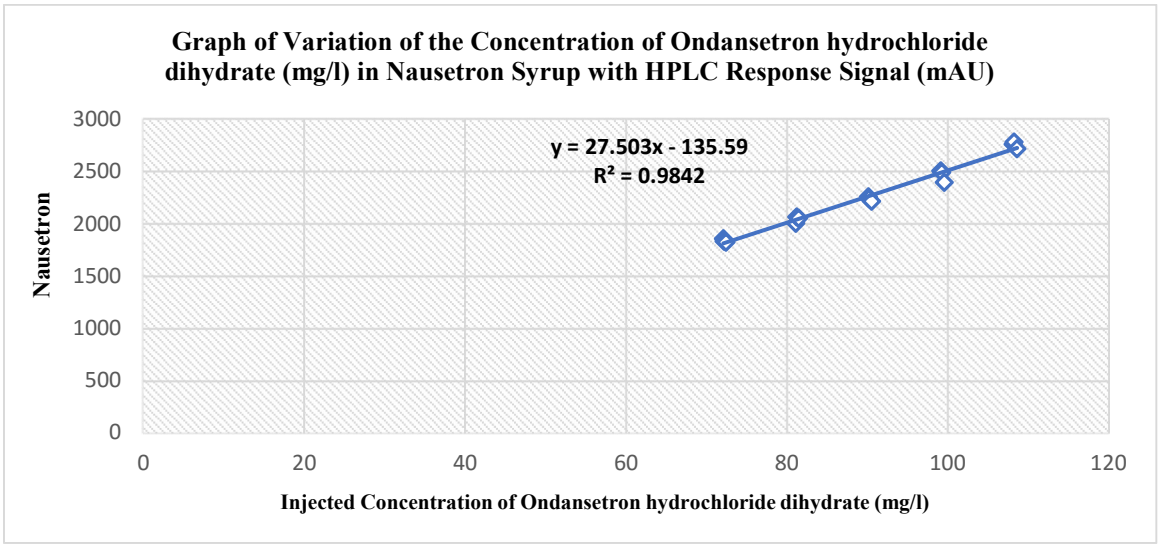


Figure 10: Graph of concentration of Ondansetron hydrochloride dihydrate against the HPLC response signal in Nausetron Syrup.

The data obtained from the study of linearity study indicates that Ondansetron hydrochloride dihydrate has a coefficient of determination and correlation of 0.9842 and 0.9921 respectively in the reconstituted syrup (dosage form) as indicated by the regression of the validation standards, and a coefficient of determination and correlation of 0.9838 and 0.9919 respectively in samples containing no matrix (API alone) as indicated by the calibration standards, all within acceptable limits.

From the study of precision, the coefficients of variation of repeatability and reproducibility are of 0.22 and 0.40 respectively, both less than 2% and within in recommended limits. From the study of accuracy (apparent recovery), the 95% confidence interval of the mean apparent recovery ranges from 95.61-100.89% and thus includes 100%. The y-intercept for Ondansetron hydrochloride dihydrate in the syrup is not significantly different from zero. The minimum and maximum concentrations of API in the dosage form bounding linearity are 76.24 and 121.75%. It can therefore be concluded that there is a linear correlation between the concentration of the ingredient, Ondansetron hydrochloride dihydrate in Ondansetron hydrochloride 4mg/5ml (Nausetron) syrup and the HPLC detector response signal with the response function passing through the origin, for a range of concentration from 76.24% to 121.75%.

DOCUMENTATION

Documentation of system suitability is accomplished by summarizing data on reproducibility, efficiency, tailing and resolution for the replicate injections. Results can also be used to troubleshoot the method. Method validation results stored in a relational database can be compared and summarized on a peak-by-peak or system-by-system basis to provide additional feedback necessary to determine system performance. No sample analysis is acceptable unless system suitability specifications have been met (Boudis H, 2015).

The report on linearity should include the slope of the regression line, the correlation coefficient the y-intercept and the residual sum of squares. The report on specificity for chromatographic methods should include representative chromatograms with well labelled peaks, resolution, plate count (efficiency), and tailing factor. Peak purity tests using advanced detection such as photodiode array (PDA) or mass spectrometry should be used to show that the response is not due to more than one component. Accuracy is reported as the percent recovery of the known, added amount, or as the difference between the mean and true value with confidence intervals. Precision is expressed as the standard deviation or the relative standard deviation (coefficient of variation) for a statistically significant number of measurements and confidence interval. Statistical tables, bar charts, and other types of graphs are commonly used to document precision. Express the LOD as the concentration of the analyte. Document and support the method used to determine LOD. An appropriate number of samples should be analysed at the limit to validate the level. In practice, it is almost never necessary to determine the actual LOD. Instead, the detection limit is shown to be sufficiently low (for example, 0.1%) to be able to reliably detect at the level specified. Express LOQ as a concentration, with the precision and accuracy of the measurement. Document and support the method used to determine LOQ. An appropriate number of samples should be analysed at the limit to validate the level. In practice, it is almost never necessary to determine the actual LOQ. Instead, LOQ is shown to be sufficiently low (e.g. 0.1%) to be able to reliable quantitate at the level specified (Boudis H, 2015).

Upon completion of the validation experiments, a draft report should be presented to your laboratory (in our case, JBXY Pharmaceuticals' quality control laboratory) for, evaluation, review, and comment, after which a final report is issued. Your company's development quality assurance department audits the final reports. To ensure maintenance of raw data, original data or copies should be made available at your company's development to facilitate auditing the study during its progress, and before the issuance of a final report. When the final report is completed, all original paper data, all magnetically encoded records, and a copy of the final report is retained in the archives of your company's development department (in our case JBXY Pharmaceuticals' development department). Your Standard Operating Procedure (SOP) should be included as reference. For example, in our case; JBXY Pharmaceuticals' Standard Operating Procedure, "Validation of Analytical Test Procedures," SOP Number MUJ-256-B dated 16th July 2015. A document revision summary is also indicated indicating the version of the method report. For example, this is an original version issued by JBXY Pharmaceuticals.

NOTES

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