"DEVELOPMENT AND VALIDATION OF A STABILITY INDICATING CHROMATOGRAPHIC METHODS FOR SIMULTANEOUS ESTIMATION OF SELECTED DRUGS IN THEIR DOSAGE FORMS USING DOE APPROACH"

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Title: "DEVELOPMENT AND VALIDATION OF A STABILITY INDICATING CHROMATOGRAPHIC METHODS FOR SIMULTANEOUS ESTIMATION OF SELECTED DRUGS IN THEIR DOSAGE FORMS USING DOE APPROACH"

Abstract:

The applicability of a quality by design (QbD) approach for the development of a sensitive and selective stability indicating chromatographic methods for simultaneous estimation of Ivabradine and Metoprolol in their combined dosage form & Budesonide and Levosalbutamol in their combined dosage form were investigated. Design of experiments was used for method development. Fractional Factorial Design was used to optimize the chromatographic conditions for HPLC & HPTLC method for Ivabradine and Metoprolol Combination. Central composite design (CCD) was used to optimize the chromatographic conditions for HPLC method of Budesonide and Levosalbutamol combination. Box-Behnken design was used to optimize the chromatographic conditions for HPTLC method of Budesonide and Levosalbutamol combination. The optimized methods (HPLC & HPTLC) for both the combinations produced sharp peaks with good resolution (>2). Significant degradation obtained after acidic & basic hydrolysis and in Oxidation condition for Ivabradine and Metoprolol. One Major impurity of Ivabradine was isolated and identified using mass spectroscopy. Significant degradation obtained after acidic & basic hydrolysis for Budesonide, and in acidic hydrolytic condition for Levosalbutamol. This approach can be applied to expedite method development and optimization activities in analytical laboratories.

Brief description on the state of the art of the research topic:

- Typically, method development is performed with a varying-one-factor-at-a time approach, which is a time-consuming process and can be susceptible to several factors [1]. Developing an HPLC and HPTLC method for a drug and its degradation products (DPs) is often a very complex and difficult process. Frequently, for degradation studies, an analyst can encounter several new peaks, disappearance of peaks, or a merging of two peaks. The analyst must then develop the method in an attempt to improve the resolution of the peaks to achieve reproducibility. The lengthy procedure that is characteristic of developing a method can be avoided by applying principles of quality by design (QbD) [2].
- Introduction of a risk-based quality initiative by the U.S. Food and Drug Administration [3] and International Conference on Harmonization (ICH) guidelines [4,5] has led to

increased usage of QbD in analytical method development and validation in the pharmaceutical industry. QbD demonstrates an understanding and a control of pharmaceutical products while providing opportunity for continuous improvement. Application of QbD in pharmaceutical manufacturing process development is well known. Its significance in analytical method development has recently gained importance, as it has helped achieve an in-depth understanding of the link between variables that affect the method and the performance of the method. Knowledge of risk helps reduce peril and improve control strategies [6].

- Design of experiment (DoE), a part of QbD, identifies the interaction or influence of critical parameters or factors during method development by conducting a minimum number of experiments [7]. Screening design can be used to recognize the factors that actually influence a method. Based on screening design results, optimization of the developed method can be achieved by using Response surface Methodology (RSM). Optimized methods are validated per guidelines. DoE thus aims to impart quality into the process and also establish a thorough understanding of the response of a system to set parameters. The above principles were applied to the method development. [8-12]
- Ivabradine is an antianginal agent. Ivabradine inhibit I_f channels ("funny channels") in the heart in a concentration-dependent manner without affecting any other cardiac ionic channels (including calcium or potassium). [13] Chemically Ivabradine is 3-[3-({[(7S)-3,4-dimethoxybicyclo [4.2.0] octa-1,3,5-trien-7-yl] methyl} (methyl)amino) propyl]-7,8-dimethoxy-2,3,4,5-tetrahydro-1H-3-benzazepin-2-one.[14]
- Metoprolol is selective β_1 receptor blocker. Chemically Metoprolol is bis(1-[4-(2-methoxyethyl) phenoxy]-3-[(propan-2-yl) amino] propan-2-ol); butanedioic acid. [15-17]

  The Newly developed combination of beta blocker (Metoprolol) with Ivabradine is safely and effectively treating coronary heart disease. So, this combination more widely used in Angina Pectoris. [18,19]
- Ivabradine and Metoprolol are estimated by various Methods. A stability indicating liquid chromatographic analytical method for the analysis of Ivabradine and Metoprolol in combined dosage form has been reported [20]. however, to the best of our knowledge no reports on stability indicating RP-HPLC method using DOE approach are available for simultaneous estimation of Ivabradine and Metoprolol in their combined dosage form.

- The present study was conducted to study the effect of QbD approach on the estimation of Ivabradine and Metoprolol in their combined dosage form.
- Budesonide is a corticosteroid. It acts by stopping the release of certain natural substances (like pro-inflammatory cytokines) in the body that are responsible for inflammation (swelling) in the airways. Chemically Budesonide is (11beta,16alpha)-16,17-(Butylidenebis(oxy))-11,21-dihydroxypregna-1,4-diene-3,20-dione [15-17].
- Levosalbutamol / Levalbuterol is a bronchodilator that works by relaxing the muscles in the airways and widens the airways. Chemically Levosalbutamol is 4-[(1R)-2-(tert-butylamino)-1-hydroxyethyl]-2-(hydroxymethyl)phenol. Together, they make breathing easier [15-17].
- There are many methods reported in the literature for the analysis of Budesonide and Levosalbutamol. Some chromatographic methods like HPLC and HPTLC are reported for estimation of Levosalbutamol and Budesonide in their combined dosage form [21,22]. However, to the best of our knowledge, no reports on stability-indicating HPLC and HPTLC methods using the DOE approach are available for simultaneous estimation of Budesonide and Levosalbutamol in their combined dosage form.

Definition of the Problem:

- Routine Monothetic analysis (change "one factor at a time") is used which is time-consuming and does not completely demonstrate the flexibility of an analytical method. Design of experiment (DoE), a part of QbD, identifies the interaction or influence of critical parameters or factors during method development by conducting experiments. The application of QbD to the development of an analytical method can ensure the robustness and reproducibility of a method when used under different input conditions. Forced Degradation (FD) studies help to predict degradation pathway of drugs and differentiate degraded products. Intrinsic stability of a drug substance in formulation can be determined.
- Literature review reveals that no reports on stability indicating HPLC and HPTLC method using DOE approach are available for simultaneous estimation of Ivabradine and Metoprolol in their combined dosage form & Budesonide and Levosalbutamol in their combined dosage form.
- So, it was thought of interest to develop and validate stability indicating HPLC and HPTLC method using DOE approach for simultaneous estimation of Ivabradine and

Metoprolol in their combined dosage form & Budesonide and Levosalbutamol in their combined dosage form.

Objectives and Scope of Work:

Objectives:

- To develop stability indicating HPLC and HPTLC method using DOE approach for simultaneous estimation of Ivabradine and Metoprolol succinate in their combined dosage form.
- To develop stability indicating HPLC and HPTLC method using DOE approach for simultaneous estimation of Budesonide and Levosalbutamol in their combined dosage form.
- To study the degradation profile of the above combinations.

✓ Hydrolytic (Acid and Base degradation)

✓ Oxidation

✓ Photolytic Degradation

✓ Thermal Degradation

✓ Neutral Degradation

- To validate the developed method according to ICH Guideline Q2(R1)
- Application of newly developed method in routine analysis for estimation of Ivabradine and Metoprolol succinate in their combined dosage form & Budesonide and Levosalbutamol in their combined dosage form.

Scope of Work:

Statistical Design of Experiments (DOE) allows to study the effect of different factors on the analysis of active ingredients by the selected method. Statistical evaluation of experimental results can be done by software. By using DOE a limited (and small) number of experiments have to be performed, so it saves the time. Rt can be predicted and developed method can be fully optimized. Forced Degradation (FD) studies help to predict degradation pathway of drugs and differentiate degraded products. Intrinsic stability of a drug substance in formulation can be determined.

Original contribution by the thesis:

Stability indicating Chromatographic methods (i.e., RP-HPLC and HPTLC) were developed and validated for the simultaneous estimation of Ivabradine and Metoprolol in their combined dosage form & Budesonide and Levosalbutamol in their combined dosage form using DOE approach. One Major Impurity of Ivabradine was isolated and identified.

The entire work in this synopsis, is the original work.

Methodology of Research and Results:

Materials and Reagents:

Ivabradine and Metoprolol were procured as gift sample from Torrent Pharmaceuticals (Ahmedabad, Gujarat) and Intas Pharmaceuticals (Ahmedabad, Gujarat) respectively. Budesonide and Levosalbutamol were procured as a gift sample from Piramal Pharmaceuticals (Ahmedabad, Gujarat) and Lupin Pharmaceuticals (Ahmedabad, Gujarat) respectively. All other reagents and solvents used were of AR grade and were purchased from Merck Chemicals, India. Instruments and Software used were mentioned in table-1.

Table 1: Instrumentation and software:

Instruments	**Company**
Modular HPLC	
Pump: LC-20AD×2 units Detector: SPD-20AV Auto Sampler: SIL-20AC HT Column Oven: CTO-10AS VP	Shimadzu, Japan
HPLC column: Shim-Pack Solar C_{18} 5µm, 250 × 4.6 mm	Shimadzu, Japan
Lab solution software Version 1.25	Shimadzu, Japan
Design-Expert trial	Stat-Ease Inc., Minneapolis
HPTLC	
Linomat 5 applicator	Camag, Switzerland
Micro-syringe (Linomat syringe 659.0014, Hamilton-Bonaduz Schweiz)	Camag, Switzerland
Pre-coated silica gel 60 $F_{254;}$ 100µm thickness HPTLC aluminium plates	Merck, Germany
Twin trough chamber	Camag, Switzerland
UV chamber and TLC scanner 4	Camag, Switzerland
visionCATS version 2.5.18262.1 software	Camag, Switzerland

Development and Validation of stability indicating HPLC method for Ivabradine and Metoprolol in their combined dosage form using DOE approach:

DoE is an approach that enables scientists to evaluate the effect and interactions of a number of variables on an output simultaneously using a limited number of experiments. In order to meet predefined TAP (target analytical profile) objectives, outputs or responses such as retention time, peak tailing, Theoretical plates and resolution between Ivabradine and Metoprolol were identified as Critical Quality Attributes (CQA) for this study. Fractional Factorial design was used to optimize chromatographic condition. Systematic and simultaneous examination of three key components *viz.*, composition of the mobile phase, Flow rate and column temperature were undertaken for optimization of chromatographic conditions using software. Preliminary trials were conducted to identify significant factor affecting response, then derived data was modelled and a number of chromatographic conditions were predicted based on that data, were identified and evaluated. This approach to optimisation was selected after considering all method attributes and based on the assumption that the factors investigated would be reliable, thereby limiting the amount of work required to demonstrate the robustness of the analytical method. Chromatographic conditions are mentioned in table-2, Degradation conditions are mentioned in table 3, Summary of developed analytical method are mentioned in table-4 and %degradation for both HPLC & HPTLC methods are mentioned in table-6

Table 2: Chromatographic condition

Parameters	**Condition**
Mobile Phase	Acetonitrile: Water (pH 4 adjusted with Ortho phosphoric acid) (15:75 v/v)
Stationary Phase	Shim-Pack Solar C_{18} 5µm, 250 × 4.6 mm
Flow rate	0.9 mL/min
Detection wavelength	220 nm
Column temperature	45^0C
Run time	15 min

Table 3: Forced Degradation study

Condition	**Degradant (Strength)**	**Temperature (^{0}C)**	**Duration (h)**	**Neutralization**
Acid	1 N HCl	70	6	1 N NaOH
Base	1 N NaOH	70	6	1 N HCl
Oxidation	6% H_2O_2	Room temperature	24	-
Dry Heat	-	70	24	-
Photo-Degradation	Methanol	30℃±2℃ Relative humidity: 35%±5 % UV Exposure: 200 Watt/m^2 Visible light: 6×10^6 Lux h	-	-

Table 4: Summary of Developed Stability Indicating HPLC Method

Sr. No.	**Parameters**		**Result**	
			Ivabradine	**Metoprolol**
1	Linearity and Range (µg/mL)		4 - 24	20-120
2	Regression equation		y = 2498.8x - 2287	y = 1476.5x - 1001.7
3	Regression Coefficient		0.9998	0.9998
4	Intraday Precision (%RSD, n=3)		0.43-0.63	0.45-0.68
5	Interday Precision (%RSD, n=3)		0.77-0.88	0.68-0.91
6	Repeatability (%RSD, n=3)		0.50	0.57
7	Accuracy (%Recovery, n=3)		100.28-100.42	99.40-100.29
8	LOD (µg/mL)		0.81	1.63
9	LOQ (µg/mL)		2.48	4.86
10	%Assay (n=3)	IVA Met XL 5mg/25mg Tablet	100.19	99.93
		IVA Met XL 5mg/50mg Tablet	99.95	100.01

Development and Validation of stability indicating HPTLC method for Ivabradine and Metoprolol in their combined dosage form using DOE approach:

- DOE was applied in development part in which Fractional factorial design was used to study the effect of factors on the R_f value of drugs. Systematic and simultaneous examination of three key components *viz.*, Concentration of Glacial acetic acid, Chloroform, Methanol, Saturation time were undertaken for optimization of chromatographic conditions using software. Twenty-five experimental runs were performed to optimize the chromatographic conditions. Aluminium sheets precoated with silica gel 60 F_{254} were used as the stationary phase. The optimized mobile phase composition was found to be Methanol: Chloroform: Ammonia: Glacial Acetic acid (6:2:0.15:0.2 v/v/v/v) and saturation time 20 min., were quantified by densitometric analysis at 282 nm. Moreover, drugs were subjected to acid and alkali hydrolysis, oxidation, thermal, and photodegradation (which is same as HPLC method table-3). Summary of developed analytical method are mentioned in table-5

Table 5: Summary of Developed Stability Indicating HPTLC Method

Sr. No.	**Parameters**		**Result**	
			Ivabradine	**Metoprolol**
1	Linearity and Range (µg/mL)		200-1200	1000-6000
2	Regression equation		y = 4.7071x + 530.56	y = 1.3483x + 635.44
3	Regression Coefficient		0.9993	0.9994
4	Intraday Precision (%RSD, n=3)		0.44-0.69	0.53-0.77
5	Interday Precision (%RSD, n=3)		0.70-0.90	0.78-0.98
6	Repeatability (%RSD, n=3)		0.73	0.94
7	Accuracy (%Recovery, n=3)		99.46-100.51	99.47-100.72
8	LOD (µg/mL)		21.07	89.76
9	LOQ (µg/mL)		63.85	272.02
10	%Assay (n=3)	IVA Met XL 5mg/25mg Tablet	100.34	100.04

		IVA Met XL 5mg/50mg Tablet	100.44	99.95

Table 6: Comparison data of % degradation by HPLC and HPTLC method:

Sr. No.	Condition	% Degradation			
		Ivabradine		Metoprolol	
		HPLC	HPTLC	HPLC	HPTLC
1	Acidic	4.416	4.23	2.08	2.12
2	Basic	5.264	5.314	2.3	2.41
3	Oxidation	3.902	3.891	2.2	2.31

Development and Validation of stability indicating HPLC method for Budesonide and Levosalbutamol in their combined dosage form using DOE approach:

Central composite design (CCD) was used to optimize chromatographic condition. Retention time, peak tailing, Theoretical plates and resolution between Budesonide and Levosalbutamol were identified as Critical Quality Attributes (CQA) for this study. Systematic and simultaneous examination of three key components *viz.*, Volume of Buffer, Volume of Methanol, pH of Buffer and Flow rate were undertaken for optimization of chromatographic conditions using software. Preliminary trials were conducted to identify significant factor affecting response, then derived data was modelled and a number of chromatographic conditions were predicted based on that data, were identified and evaluated. Chromatographic conditions are mentioned in table-7, Degradation conditions are mentioned in table-8, Summary of developed analytical method are mentioned in table-9 and %degradation for both HPLC & HPTLC methods are mentioned in table-11

Table 7: Chromatographic condition

Parameters	**Condition**
Mobile Phase	Acetonitrile: Phosphate Buffer (pH 3.2 adjusted with OPA): Methanol (62:28:10 v/v)
Stationary Phase	Shim-Pack Solar C_{18} 5µm, 250 × 4.6 mm
Flow rate	0.6 mL/min

Detection wavelength	231 nm
Column temperature	40^0C
Run time	20 min

Table 8: Forced Degradation study

Condition	**Degradant (Strength)**	**Temperature (^{0}C)**	**Duration (h)**	**Neutralization**
Acid	0.05 N HCl	70	3	0.05 N NaOH
Base	0.01 N NaOH	70	3	0.01 N HCl
Oxidation	30% H_2O_2	Room temperature	15 days	-
Dry Heat	-	70	24	-
Photo-Degradation	Methanol	30°C±2°C Relative humidity: 35%±5 % UV Exposure: 200 Watt/m^2 Visible light: 6×10^6 Lux h	-	-

Table 9: Summary of Developed Stability Indicating HPLC Method

Sr. No.	**Parameters**	**Result**	
		Budesonide	**Levosalbutamol**
1	Linearity and Range (µg/mL)	8-48	10-60
2	Regression equation	y = 26731x - 8967	y = 34764x - 28104
3	Regression Coefficient	0.9998	0.9993
4	Intraday Precision (%RSD, n=3)	0.43-0.57	0.34-0.59
5	Interday Precision (%RSD, n=3)	0.90-1.07	1.09-1.24
6	Repeatability (%RSD, n=3)	0.97	0.82

7	Accuracy (%Recovery, n=3)		99.45 – 99.62	99.40-99.83
8	LOD (µg/mL)		0.45	0.65
9	LOQ (µg/mL)		1.38	1.99
10	%Assay (n=3)	IVA Met XL 5mg/25mg Tablet	99.70	99.71
		IVA Met XL 5mg/50mg Tablet	99.83	99.68

Development and Validation of stability indicating HPTLC method for Budesonide and Levosalbutamol in their combined dosage form using DOE approach:

- DOE was applied in development part in which Box-Behnken design was used to study the effect of factors on the R_f value of drugs. Twenty-five experimental runs were performed to optimize the chromatographic conditions. Aluminium sheets precoated with silica gel 60 F_{254} were used as the stationary phase. The optimized mobile phase composition was found to be Toluene: Ethyl acetate: Methanol: Ammonia (4:1.8:1.8:0.2) and saturation time 20 min, were quantified by densitometric analysis at 231 nm. Moreover, drugs were subjected to acid and alkali hydrolysis, oxidation, thermal, and photodegradation (which is same as HPLC method table-8). Summary of developed analytical method are mentioned in table-10

Table 10: Summary of Developed Stability Indicating HPTLC Method

Sr. No.	**Parameters**	**Result**	
		Budesonide	**Levosalbutamol**
1	Linearity and Range (µg/mL)	221-1120	280-1400
2	Regression equation	y = 5.0045x + 15	y = 4.8577x + 94.833
3	Regression Coefficient	0.9993	0.9995
4	Intraday Precision (%RSD, n=3)	0.48-0.88	0.60-0.72
5	Interday Precision (%RSD, n=3)	0.84-1.13	0.74-1.02
6	Repeatability (%RSD, n=3)	0.82	0.67
7	Accuracy (%Recovery, n=3)	99.78-99.45	99.13-99.79
8	LOD (µg/mL)	13,14	10.89

9	LOQ (µg/mL)		39.84	33.02
10	%Assay (n=3)	IVA Met XL 5mg/25mg Tablet	99.65	99.86
		IVA Met XL 5mg/50mg Tablet	100.19	100.26

Table 11: Comparison data of % degradation by HPLC and HPTLC method:

Sr. No.	Condition	% Degradation			
		Budesonide		Levosalbutamol	
		HPLC	HPTLC	HPLC	HPTLC
1	Acidic	14.6	13.72	45.98	46.1
2	Basic	17.4	17.49	-	-

Achievements with respect to Objectives:

- Stability indicating Chromatographic methods (i.e. RP-HPLC and HPTLC) were developed and validated for the simultaneous estimation of Ivabradine and Metoprolol using DOE approach. The proposed methods were applied in marketed formulations of Ivabradine and Metoprolol (IVA Met XL 5mg/25mg Tablet, IVA Met XL 5mg/50 mg Tablet). Major Impurities of Ivabradine were isolated and identified using Mass Spectroscopy.
- Stability indicating Chromatographic methods (i.e. RP-HPLC and HPTLC) were developed and validated for the simultaneous estimation of Budesonide and Levosalbutamol. The proposed methods were applied in marketed formulations of Budesonide and Levosalbutamol [**Budesal respules 0.5 mg** (Budesonide 0.5 mg, Levosalbutamol 1.25 mg), **Budesal respules 1 mg** (Budesonide 1 mg, Levosalbutamol 1.25 mg)].

Conclusion

- Stability indicating Chromatographic methods (i.e. RP-HPLC and HPTLC) were developed and validated for the simultaneous estimation of Ivabradine and Metoprolol

using DOE approach. The proposed methods were applied in marketed formulations of Ivabradine and Metoprolol (IVA Met XL 5mg/25mg Tablet, IVA Met XL 5mg/50 mg Tablet).

- Ivabradine and Metoprolol are found to be stable in Thermal and Photolytic condition. Significant degradation observed in acidic, basic and Oxidation condition.
- Major Impurities of Ivabradine were isolated and identified using Mass Spectroscopy.
- Stability indicating Chromatographic methods (i.e. RP-HPLC and HPTLC) were developed and validated for the simultaneous estimation of Budesonide and Levosalbutamol. The proposed methods were applied in marketed formulations of Budesonide and Levosalbutamol [Budesal respules 0.5 mg (Budesonide 0.5 mg, Levosalbutamol 1.25 mg), Budesal respules 1 mg (Budesonide 1 mg, Levosalbutamol 1.25 mg)].
- Significant degradation observed in acidic and basic condition for Budesonide, Significant degradation observed in acidic condition for Levosalbutamol
- Validated Method was found to be simple, accurate, robust and reproducible. There was no interference of any excipients in the determination of drug from marketed formulation.
- Both the Methods were statistically validated using Student t-test. The results indicated that both the methods are equally sensitive, reliable and can be use in routine analysis.

Publication:

1. **Noopur Gandhi**, Sindhu Ezhava, "Stability indicating Analytical Method Development using Quality by Design (QbD) approach for simultaneous estimation of Ivabradine and Metoprolol", *Research Journal of Pharmacy and Technology*, 2021, Vol: 14, Issue: 11, ISSN 0974-3618 (Accepted)
2. **Noopur Gandhi**, Sindhu Ezhava, "Ivabradine and Metoprolol: A review of analytical methods for pharmaceutical quality control and monitoring", *International Journal of Pharmaceutical Sciences and Research,* December 2021, Vol:12, Issue:12 (Accepted)

Communicated:

1. **Noopur Gandhi**, Sindhu Ezhava, "Stability indicating Analytical Method Development using Quality by Design (QbD) approach for simultaneous estimation of Budesonide and Levosalbutamol" Communicated to *Journal of AOAC International*. **Current Status-** review is in process

Publications arising from the thesis:

Sr. No.	Probable Title	Probable Journal
1.	Stability indicating High-Performance Thin-Layer Chromatographic Method for simultaneous estimation of Ivabradine and Metoprolol using Quality by Design (QbD) approach	Analytical chemistry letters
2.	Development and Validation of a Stability indicating HPLC Method for simultaneous estimation of Budesonide and Levosalbutamol using Design of Experiment (DOE) Approach	Journal of Liquid Chromatography & Related Technologies

Patents (if any) ---------- NA----------

References:

1. Monks KE, Rieger HJ, Molnar I (2011) Expanding the term "Design Space" in high performance liquid chromatography, *J. Pharm. Biomed. Anal.*, Vol. 56(5), 874–879. ISSN: 2473-0831
2. Debrus B, Guillarme D, Rudaz S (2013) Improved quality-by-design compliant methodology for method development in reversed-phase liquid chromatography, *J. Pharm. Biomed. Anal.*, Vol. 84, 215–223. ISSN: 0731-7085
3. Department of Health and Human Services, U.S. Food and Drug Administration (2004) Pharmaceutical cGMPs for the 21st Century—A Risk-Based Approach, https://www.fda.gov/downloads/drugs/developmentapprovalprocess/manufacturing/questionsandanswersoncurrentgoodmanufacturingpracticescgmpfordrugs/ucm176374.pdf

4. International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use, Topic Q9: Quality Risk Management, ICH, Geneva, Switzerland (2005) https://www.ich.org/fileadmin/Public_Web_Site/ICH.../Q9/.../Q9_Guideline.pdf
5. International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use, Topic Q10: Pharmaceutical Quality System, ICH, Geneva, Switzerland (2008) https://www.ich.org/fileadmin/Public_Web_Site/ ICH.../Q10/.../ Q10_Guideline.pdf
6. Ye C., Terfloth G, Li Y, Kord A (2009) A systematic stability evaluation of analytical RP-HPLC columns *J. Pharm. Biomed. Anal.,* Vol. 50(3), 426–431. ISSN: 0731-7085
7. Debrus B, Lebrun P, Ceccato A, et al. Application of new methodologies based on design of experiments, independent component analysis and design space for robust optimization in liquid chromatography, *Anal. Chim. Acta,* Vol. 691(1-2), 33–42. ISSN: 0003-2670
8. Hibbert DB (2012) Experimental design in chromatography: a tutorial review, *Analyt. Technol. Biomed. Life Sci.,* Vol. 910, 2–13. ISSN: 1873376X, 15700232
9. Hendriks MM, De Boer JH, Smilde AK, et al. (1992) Multicriteria decision making, *Chemometr. Intell. Lab. Syst.,* Vol. 161, 75–191. ISSN: 0169-7439
10. Araujo PW, Brereton RG (1996) Experimental design I. Screening, *Trends Anal. Chem.,* Vol. 15, pp 26–31. ISSN: 0165-9936
11. Kuchekar AB, Pawar AP (2014) Design Space by Design of Experiments, *Int. J. Pharm. Pharm.Sci*., Vol. 6, 489–496. ISSN: 0975-1491
12. Ermer J, Nether Cote PW (2014) Method Validation in Pharmaceutical Analysis: A Guide to Best Practice, Wiley-VCH Verlag GmBH & Co., Weinheim, Germany, 2nd Ed., pp. 50–171.
13. Du XJ, Feng X, Gao XM, Tan TP, et al. (2004) I_f channel inhibitor Ivabradine lowers heart rate in mice, *British J. of Pharmacology*, Vol. 142, pp 107-112. ISSN: 1476-5381
14. Ivabradine, C27H36N2O5–PubChem. https://pubchem.ncbi.nlm.nih.gov/compound/Ivabradine, [Accessed 1/11/21]
15. Indian Pharmacopoeia (2018) Government of India Ministry of Health and Family Welfare, Published by The Indian Pharmacopoeia Commission, Ghaziabad, Vol-I, Vol-II, pp. 583, 431, 563, 2583-2593, 1413-1417, 2430-2434

16. United State pharmacopoeia and National formulary (2017) Asian Edition, The United State Pharmacopeial Convention, Rockville, USP 40 NF 35, Vol-II, Vol-III, pp. 2606-2612, 3061-3062, 5135-5146.
17. Maryadele NJ (2006) The Merck Index an Encyclopedia of chemicals drugs and biological,12th Edn; Merck Research Laboratories, UK, pp. 213, 1488, 6234
18. Ptaszynski P, Kaczmarek K, Ruta J, et al. (2013) Ivabradine in combination with Metoprolol succinate in the treatment of inappropriate sinus tachycardia, *J. Cardiovasc. Pharm. T.*, Vol. 18(4), 338-344. ISSN: 1940-4034
19. Zarifis J, Kallistratos M and Katsivas A. (2016) Antianginal Efficacy of Ivabradine and Metoprolol Combination in Patients with Stable Angina, *Clin. Cardiol.*, Vol. 39(12), 697-702. ISSN: 2753-1201
20. Kanthale SB, Thonte SS and Mahapatra DK. (2019) Stability Indicating RP-HPLC Method for the simultaneous estimation of Ivabradine and Metoprolol in bulk and tablet formulation, *J. of Applied Pharm. Sci.,* Vol. 9(4), 137-144. ISSN: 22313354
21. Dadhich S, Sharma S, Bhandari A, Agarwal A, et al (2013) Method Development and Validation for Simultaneous Estimation of Levosalbutamol Sulphate and Budesonide in Bulk and Pharmaceutical Dosage Form by RP-HPLC, *Am. J. PharmTech Res.,* vol. 3(2), 939-947. ISSN 2249-3387
22. Prajapati PB, Marolia BP, Bodiwala KB, Vadodariya JM, Shah SA (2015) Development and Validation of HPTLC Method for Simultaneous Estimation of Budesonide and Levalbuterol Hydrochloride in their Combined Pharmaceutical Dosage Forms, *Journal of Pharmacy and Applied Sciences*, Vol. 2(1), 21-28. ISSN: 2395-6100