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Development of Analytical Method Using Classical Regression Technique

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Abstract: The study was oriented towards development of analytical method and prediction of real relationship between absorbance and concentration variables while conforming to validation parameters. Seven compositions, A1, A2, A3, B1, B2, B3, C containing different ratios of model drug (ofloxacin) and polymers (HPMC and sodium alginate) were used to prepare ofloxacin composition solutions of 5 μg mL^{-1} in Simulated Gastric Fluid (SGF, pH 1.2), buffer (pH 6.2) and Simulated Intestinal Fluid (SIF, pH 7.5). Similarly, ofloxacin solutions of 2, 4, 5, 6 and 8 μg mL^{-1} were also prepared. The method was assessed with respect to (w.r.t.) all requisite principle parameters like specificity, precision, range and inter/intra-day variations alongwith linearity/non-linearity (model generation) and balancing of the variance at various concentration levels. The specificity was notified from constant absorbance and non-shifting absorbance wavelength maxima (λ_{max}) of unspiked and spiked samples. The RSD value <5.0% for absorbance readings of various samples, prepared at different times, expressed the precision and inter/intra-day variations within the limits. The absorbance values within 0.2-0.8 for 2-8 μg mL^{-1} concentrations in different media satisfied the concentration range for analysis. For standard plots, equation $y_i = \beta_1 X_i + e_i$ (Model 2 or equation 3) was sorted as final equation on basis of model selection scheme (Scheme 1). For balancing variance, weight=1/conc.^{2} was found best on basis of weight selection scheme (Scheme 2). The classical approach predicted the real relationship between concentration and absorbance values and can be applied similarly to other spectroscopic techniques for analytical methods development.

Key words: Polynomial, HPMC, ofloxacin, UV-spectrophotometry, models, weights

INTRODUCTION

The guidance for industry on analytical procedures and method validation, provides recommendations to applicants on submitting analytical procedures, validation data and samples to support the documentation of the identity, strength, quality, purity and potency of drugs and drug products. The FDA, EU, TGA and most of other organizations requires that for development of any analytical procedure, information must include data demonstrating accuracy, precision, linearity, specificity, ruggedness, range, limit of detection and limit of quantitation (FDA, 2000; EURACHEM Guide, 1998; TGA, 2006; Thompson *et al*., 2002). The USP, ICH Q2A and Q2B address almost all of these validation parameters, although all the parameters are not needed for all types of methods by FDA, USP as well as ICH (ICH Q2A, 1995; ICH Q2B, 1996; Robinson and Lee, 1987). Any quantitative analytical technique require experiments to be conducted to ensure consistent results in concern of these parameters (Tahir *et al*., 1999). Chromatographic methods are used primarily for determination of drugs in biological fluids as well as pharmaceutical products but simple UV-spectrophotometric methods are suitable for day-to-day analysis of aqueous solutions (Amini *et al*., 2005). Where UV-spectrophotometric analyses are more convenient for frequent use, the methodology of relating response (absorbance) vs. predictor (concentration) variables may introduce huge error in estimations. The relationship between absorbance and concentration may be linear, quadratic or logarithmic, hence, direct use of straight line equation $y = \alpha+\beta X$ should be avoided. The prediction of relationship between variables may be started from a polynomic equation which exhibits relationship between absorbance and concentration variables as under:

$$y_i = \alpha+\beta_1 X_i+\beta_2 X_i^2+\beta_3 X_i^3+\beta_4 X_i^4+e_i \quad (1)$$

This equation takes care of non-linear behavior of absorbance with concentration and can also get converted to various other equations (statistical or

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mathematical models) on the basis of statistical significance of coefficients. The five different types of models or equations, acceptable with FDA, which can originate from this polynomic equations are:

- Model 1 $y_i = \alpha+\beta_1 X_i+e_i$ (2)
- Model 2 $y_i = \beta_1 X_i+e_i$ (3)
- Model 3 $y_i = \alpha+\beta_1 X_i+\beta_2 X_i^2+e_i$ (4)
- Model 4 $y_i = \alpha X_i^{\beta} e_i$ or $\log y_i = \log \alpha+\beta \log X_i+\log e_i$ (5)
- Model 5 $y_i = \alpha e^{\beta xi} e_i$ or $\log y_i = \log \alpha+\beta X_i+\log e_i$ (6)

where in each model, y and x are absorbance and concentration terms. The α, β are coefficients of respective equations. The scheme for selection of appropriate coefficients has been gives as Scheme 1.

Among these models or equations, model 1 and 2 are simple straight line equations and models 4, 5 get transform to linear equations after log transformations. The model 3 is a quadratic representation of relationship between absorbance and concentration values. For accurate calibration of an instrument and generation of standard curve, only model selection is not final criteria. The variance in absorbance values is often heterogeneous at different concentration levels which introduce error in relationship prediction. Therefore, smoothing of variances is required that can be done by appropriate weight selection as per Scheme 2, starting with equation given below:

$$SD(y) = \delta_0 + \delta_1\sqrt{X} + \delta_2 x + e_i \quad (7)$$

where, SD(y) and X are standard deviation of absorbance (response) and concentration terms. The δ are coefficients as explained for polynomial equation. If in any case, $\delta_1 = \delta_2 = 0$, the SD (y) becomes independent of concentration and variance is considered constant throughout the concentration range. In such cases there is no need of weight or weight becomes equal to unity. For cases where $\delta_1 \neq \delta_2 \neq 0$, the choice of appropriate weight is decided on the basis of Sum of Square (SS) values as given in Scheme 2. Where; SS ($\delta_2|\delta_0$, δ_1) means SS due to inclusion of the $\delta_2 X$ when δ_0 and $\delta_1\sqrt{X}$ already exist in Eq. 7. Similarly SS (δ_1 $|\delta_0$, δ_2) indicates SS due to inclusion of the $\delta_1\sqrt{X}$ when δ_0 and $\delta_2 X$ already exist in Eq. 7. So on basis of Scheme 2, weight = 1/conc.^{2} and 1/conc. can be selected to make variance homogeneous (Chow and Liu, 1995; Shahzad *et al*., 2003).

In this experiment, a UV-visible spectrophotometric method has been developed to describe the processing of data in a way to relate absorbance and concentration variables as per above theory. The model drug ofloxacin is a fluoroquinolone category drug, freely soluble in water and measurable by UV-spectroscopy. Ofloxacin and other drugs that are rapidly and uniformly absorbed after oral administration and have high oral bioavailability (~100%) are good candidate for oral Extended Release (ER) dosage forms. The ofloxacin ER tablet compositions of our previous publication have been used to define various analytical parameters and absence of interference from tablet excipients (Singh *et al*., 2011).

MATERIALS AND METHODS

Materials, reagents and tablet composition: Ofloxacin (assay 99.8%) was provided by Ranbaxy, New Delhi, India. Hydroxypropyl methyl cellulose (3000 cps) and sodium alginate were purchased from S.D. Fine-chem. Ltd., Mumbai, India. Simulated Gastric Fluid (SGF) of pH 1.2 without enzymes, Simulated Intestinal Fluid (SIF) of pH 7.5 without enzymes and phosphate buffer of pH 6.2 were prepared as per USP standards. All the chemicals used were of AR grade.

The tablet composition "C" of our previous publication consists of ofloxacin: HPMC: sodium alginate in 5:1:1 ratio (300 mg Ofloxacin, 60 mg HPMC, 60 mg sodium alginate) alongwith minor quantities of other assisting substances like 3 mg magnesium stearate and 2 mg of talc (Singh *et al*., 2011). Various other ofloxacin compositions (A1, A2, A3, B1, B2, B3) were also prepared for assessing the specificity of the analytical method. The compositions A1, A2, A3 were containing ofloxacin: HPMC in 2.5 : 1, 4 : 1, 5 : 1 ratios; while B1, B2, B3 were containing ofloxacin: sodium alginate in 2.5 : 1, 4 : 1, 5 : 1 ratios. The amounts of ofloxacin and polymers in all the compositions were used as per above ratios for an average of 425 mg final tablet mixture.

Ofloxacin and ofloxacin composition solutions: The ofloxacin solutions were prepared by dissolving ofloxacin in SGF, buffer and SIF. Similarly, ofloxacin composition solutions were prepared by dissolving ofloxacin compositions A1, A2, A3, B1, B2, B3 and C in SGF, buffer and SIF. The solutions prepared were stock ofloxacin and stock ofloxacin composition solutions of 1 mg mL^{-1} ofloxacin concentration.

Apparatus/ lab conditions: UV-visible spectrophotometer -Beckman DU 640B, USA, Quartz cuvette of 1 cm path

length were used for analysis. The pipettes and volumetric flasks were certified class A apparatus, calibrated at 27°C. The make of weighing balance was Afcoset ER- 182A, Mumbai, India. All the measurements were carried out at a lab temperature of 27±2°C.

Specificity of the method: Spiking experimental technique was used for explaining specificity. The spiked samples of ofloxacin: HPMC, ofloxacin: sodium alginate and ofloxacin: HPMC: sodium alginate were analyzed side by side with unspiked samples to demonstrate effect on maximum absorbance wavelength (λ_{max}) (Amini *et al*., 2005). The unspiked samples of concentration 5 μg mL^{-1} were prepared from stock solutions of ofloxacin (1 mg mL^{-1}) after diluting with SGF, buffer and SIF. Similarly, spiked samples of 5 μg mL^{-1} concentration were prepared from stock ofloxacin composition solutions with SGF, buffer and SIF. These standard ofloxacin composition solutions were named A1, A2, A3, B1, B2, B3 and C as per their original source of ofloxacin compositions. The samples were scanned in the range of 200-400 nm to determine λ_{max}. The absorbance scans and absorbance values of these standard ofloxacin composition solutions were compared with that of the standard ofloxacin solutions. The absence of interference from polymers was decided on the basis of;(1) absence of change (<5 nm) in λ_{max} for standard ofloxacin solutions and standard ofloxacin composition solutions in their respective media, and (2) less than 5.0% Relative Standard Deviation (RSD) in absorbance values for standard ofloxacin solutions and standard ofloxacin composition solutions.

Precision, range and stability of method: Ofloxacin stock solutions and ofloxacin composition stock solutions were diluted with respective media to get five concentrations between 2-8 μg mL^{-1} range. Afresh solutions of each concentration were prepared and analyzed at 0th, 4th and 8th h of the day. The method was repeated for three consecutive days with preparation of new solutions each day to address all the aspects of intra- and inter-day variations. The precision, range and stability of the method were established on the basis of RSD values. In most of the cases the method is said to be precise and stable if RSD is found to be <5.0% for response variable. In contrast, the RSD values up to 10% has also been reported in literature for analysis of ofloxacin in plasma and other biological fluids (Amini *et al*., 2005).

Model selection and weight selection: The choice of appropriate model or equation was done to describe actual relationship between absorbance and concentration variables. The absorbance values were related to concentrations as per polynomial regression in Microsoft Excel spreadsheet. Depending upon coefficients (α, β) values obtained from regression statistics, the polynomial equation was reduced to appropriate model as per Scheme 1. In most of the analytical methods, the relationship between absorbance and concentration reduces to either model 1 or model 2. In rare cases the relationship may lie among model 3, 4 or 5. The selection among higher models i.e., model 4 and 5, needs further assessment of Residual Sum of Square (RSS) values before finalization of either model.

As for model selection, the weight selection was also decided on the basis of coefficients (δ_1 and δ_2) values obtained from regression analysis on standard deviation (SD(y)) vs. concentration in Microsoft Excel spreadsheet. The selection of appropriate weights has been given in Scheme 2. The maximum acceptance level for both model selection and weight selection was kept at a p-level of 5.0%.

RESULTS AND DISCUSSION

Specificity of the method: As mentioned, the final compliance with specificity was done on basis of; a) insignificant change in λ_{max} of spiked and unspiked solutions and b) consistent value of absorbance for spiked and unspiked solutions. The λ_{max} of unspiked ofloxacin solutions was found to be 293±2, 286±3, 288±2 nm in SGF, buffer and SIF. The λ_{max} of spiked ofloxacin solutions, i.e., A1, A2, A3, B1, B2, B3 and C, were found to be lying within 292±3, 286±3, 287±4 nm in SGF, buffer and SIF (Table 1). The fluctuations were under the permissible limits and hence 293, 286 and 288 nm were considered as final λ_{max} values on an average basis for both ofloxacin solutions and ofloxacin composition solutions. The interference was also ruled out since data in Table 1 indicates that absorbance values of unspiked and spiked solutions remained within 5% RSD in all media. Therefore, the method remained specific w.r.t. ofloxacin even in the presence of excipients up to their maximum amounts utilized.

Precision, range and stability of method: Table 2 indicates that RSD of absorbance values (n = 9) at each concentration level i.e., 2, 4, 5, 6 and 8 μg mL^{-1} was <5.0%. As the solutions were prepared three times, on three consecutive days and each sample was analyzed thrice on its respective preparation day, so the inter- and intra-day variations in the data remained under 5%, which reflects precision and stability of the

Table 1: Specificity and Interference data

Media	Ofloxacin Av. Ab. (λ_{max})	A1 Av. Ab. (λ_{max})	A2 Av. Ab. (λ_{max})	A3 Av. Ab. (λ_{max})	B1 Av. Ab. (λ_{max})	B2 Av. Ab. (λ_{max})	B3 Av. Ab. (λ_{max})	C Av. Ab. (λ_{max})	Final Av. Ab.	Standard deviation	RSD
SGF	0.492 (293±2 nm)	0.497 (292±3 nm)	0.509 (292±3 nm)	0.454 (291±1 nm)	0.449 (292±2 nm)	0.465 (293±2 nm)	0.470 (291±2 nm)	0.492 (292±2 nm)	0.479	0.022	4.6
Buffer	0.478 (286±3 nm)	0.456 (285±2 nm)	0.448 (286±2 nm)	0.451 (286±2 nm)	0.468 (285±1 nm)	0.482 (285±2 nm)	0.436 (285±3 nm)	0.449 (286±2 nm)	0.459	0.016	3.5
SIF	0.388 (288±2 nm)	0.406 (288±3 nm)	0.371 (287±3 nm)	0.390 (286±3 nm)	0.384 (288±3 nm)	0.376 (288±1 nm)	0.360 (287±2 nm)	0.363 (288±3 nm)	0.380	0.015	4.0

Av. Ab.: Average absorbance (n = 3), RSD: Relative standard deviation

Table 2: Precision, stability, range and weight data

	SGF (pH≈1.2)					Buffer (pH = 6.2)					SIF (pH = 7.5)				
Conc. ($\mu g\ mL^{-1}$)	Av. Ab. (n = 9)	SD	RSD	Weight $(mL/\mu g)^2$	Cal. Ab	Av. Ab. (n = 9)	SD	RSD	Weight $(mL/\mu g)^2$	Cal. Ab.	Av. Ab. (n = 9)	SD	RSD	Weight $(mL/\mu g)^2$	Cal. Ab.
0	0.000	0.000	0.000	0.000	0.007	0.000	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000	0.000
2	0.198	0.009	4.502	0.250	0.197	0.188	0.007	3.918	0.250	0.186	0.158	0.007	4.608	0.250	0.158
4	0.382	0.011	2.937	0.063	0.387	0.352	0.015	4.374	0.063	0.366	0.325	0.015	4.468	0.063	0.316
5	0.482	0.016	3.285	0.040	0.482	0.454	0.022	4.792	0.040	0.456	0.390	0.019	4.940	0.040	0.395
6	0.581	0.028	4.838	0.028	0.577	0.549	0.024	4.317	0.028	0.546	0.473	0.023	4.892	0.028	0.474
8	0.770	0.037	4.763	0.016	0.768	0.742	0.032	4.289	0.016	0.726	0.631	0.024	3.823	0.016	0.633

Av. Ab.: Average absorbance, SD: Standard deviation, RSD: Relative standard deviation, Cal. Ab.: Calculated absorbance

Table 3: Coefficients of polynomial and weight selection scheme

	SGF (pH≈1.2)			Buffer (pH = 6.2)			SIF (pH = 7.5)		
Coeff.	Values	SE	p-value	Values	SE	p-value	Values	SE	p-value
α	0.000	0.002	0.989	0.000	0.006	0.989	0.000 (0.000)	0.008 (0.007)	0.989 (0.925)
β_1	0.113	0.005	0.029	0.117	0.016	0.046	0.071 (0.083)	0.022 (0.008)	0.187 (0.010)
β_2	-0.011	0.003	0.188	-0.018	0.010	0.321	0.007 (-0.001)	0.013 (0.002)	0.708 (0.699)
β_3	0.002	0.001	0.190	0.004	0.002	0.324	-0.001 (0.000)	0.003 (0.0002)	0.678 (0.733)
β_4	0.000	0.000	0.192	0.000	0.000	0.337	0.000$^{\$}$ (--)	0.000$^{\#}$ (--)	0.666 (--)
δ_0	0.000	0.005	0.931	0.000	0.001	0.933	-0.003	0.001	0.014
δ_1	-0.006	0.005	0.041	0.002	0.001	0.039	0.002	0.000	0.003
δ_2	0.007	0.003	0.044	0.004	0.001	0.007	0.003	0.000	0.000

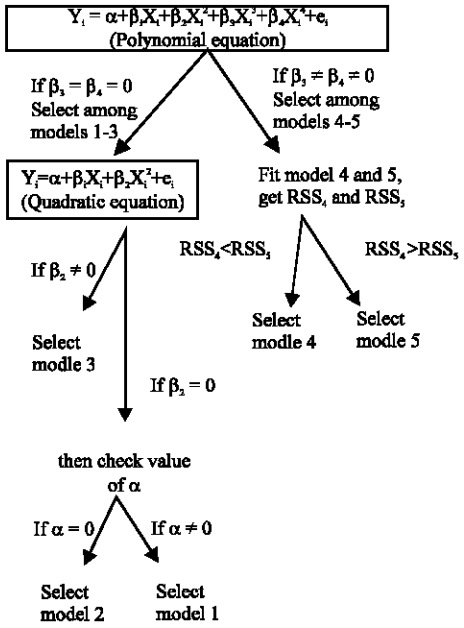
Coeff.: Coefficients, SE: Standard error, $^{\$}$Actual value is 0.000100, $^{\#}$Actual value is 0.000173

analytical method. The absorbance readings were lying between 0.2-0.8, so concentration range of 2-8 $\mu g\ mL^{-1}$ can be considered as appropriate for the spectrophotometric analysis of ofloxacin.

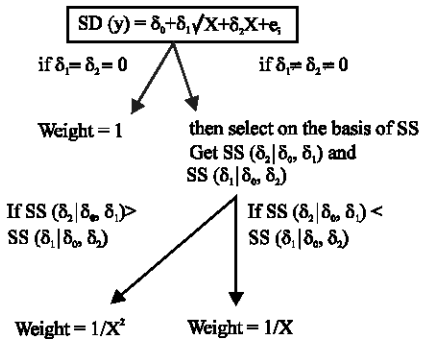
Model selection: As per Scheme 1, all five models can be derived from single polynomial Eq. 1 on the basis of values of different coefficients. The coefficient values were decided on the basis of Standard Error (SE) and p-values. The p-value is the probability of obtaining the estimated value of the coefficient if the actual coefficient is zero. The smaller the p-value, the more significant is the parameter and less likeliness of the coefficient value to be equal to zero. The decision for considering any coefficient equal to zero on basis of p-value is applicable only when SE value for that coefficient is smaller than coefficient's own value. If the SE is more than coefficient's value then this indicates that coefficient is showing more fluctuation and must be considered zero directly or independently of the p-value. In SGF (Table 3), value of α is 0.000 (not an absolute zero, rounded to three decimal places) and its SE is 0.002, so error is more as compared to α value, hence its value was considered equal to zero irrespective of the p-value. For β_1, β_2, β_3 and β_4 the SE values were less than coefficients values, so, p-values were considered for these coefficients. The p-value <0.05 was found only for β_1 hence β_2, β_3 and β_4 were considered equal to zero and polynomial equation reduced to $y_i = \beta_1 X_i + e_i$ (Model 2) equation. Therefore, model 2 was finalized for SGF case according to Scheme 1.

For buffer (Table 3), the β_1 was significant as its SE was less than its own value and p-value <0.05. The α was considered zero as its SE was more from its own value and β_2, β_3 and β_4 were not considered due to non-significant p-values i.e., >0.05. Thus model 2 was finalized for buffer case.

For SIF (Table 3), the SE values were larger for β_2, β_3 and β_4 than coefficients own values and p-value was non-significant for β_1, so all of the coefficients were considered equal to zero. But in this way absorbance became independent of concentration term, which is not true in reality. Such type of results appears mainly when



Scheme 1: Selection of Statistical Models for Standard Curves (α is Intercept, β is Slope and e_i is Independent Variable)



Scheme 2: Selection of Weight for Regression Model (Where SS $(\beta_2 |\beta_0, \beta_1)$ denotes Regression Sum of Squares due to inclusion of the β_2X when β_0 and β_1X already exists in model)

data is related in a linear manner but it is tried to fit to a higher polynomial relationships. The solution to such a problem is starting with reduced polynomial equation i.e., $y_i = \alpha+\beta_1X_i+\beta_2X_i^2+\beta_3X_i^3$. The new coefficient values for reduced equation have been given in parenthesis in Table 3. Among the coefficients, β_1 is the only coefficient having high value than SE and significant p-value. So, in SIF, again model 2 was the final outcome explaining the relationship between absorbance and concentration values.

Table 4: Regression sum of square values for weight selection scheme

	Regression SS values		
Parameters in Eq. 7	SGF	Buffer (pH 6.2)	SIF
δ_0, $\delta_1\sqrt{X}$, δ_2X	0.00090	0.00067	0.00045
δ_0, $\delta_1\sqrt{X}$	0.00075	0.00061	0.00042
δ_0, δ_2X	0.00089	0.00067	0.00043

SS: Sum of square

As the model 2 has been the model of choice in all pH conditions, the relationship between absorbance and concentrations can be declared linear and following $y_i = \beta_1X_i+e_i$ (model 2) equation in all cases.

Weight selection: The Scheme 2 of weight selection was applied on SD(y) and concentration values. The different coefficients (δ_1 and δ_2) were related to their levels of significance on basis of the same methodology as used for model selection. The coefficient values, SE and p-values have been given in Table 3. As the values of coefficients δ_1 and δ_2 were not equal to zero i.e., $\delta_1 \neq \delta_2 \neq 0$ in either of SGF, buffer and SIF, so, selection of weights was done on the basis of SS values. In Table 4, it is clear that SS due to inclusion of δ_2X i.e., $SS(\delta_2|\delta_0, \delta_1)$ was more as compared to SS due to the inclusion of $\delta_1\sqrt{X}$ i.e., $SS(\delta_1|\delta_0, \delta_2)$ in all the cases, so the weight = $1/conc.^2$ was chosen for making variance homogeneous.

CONCLUSION

In the present study a validated analytical procedure for ofloxacin determination in SGF, buffer and SIF has been developed. Classical regression methodology helped in prediction of best relationship between absorbance and concentration values alongwith balancing of variance heterogeneity. Further, the general statistical methodology also helped in screening of analytical method with respect to various validation parameters like specificity, precision, range, stability and inter/ intra-day viability. Therefore, classical regression alongwith general statistics is beneficial in development of appropriate analytical method.

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