Journal of Pharmaceutical Research Vol. 8, No. 2, April 2009 : 70-75.

AREA UNDER ABSORBANCE CURVE vs. CONCENTRATION FOR UV-SPECTROPHOTOMETRIC ANALYSIS OF INSULIN IN DIFFERENT pH CONDITIONS

Singh Jasbir [a,b] and Singh Gajendra* [a,b]

[a] Department of Pharmaceutical Sciences, Guru Nanak Dev University, Amritsar -143 005, Punjab. India.

[b]Department of Pharmacy, Pt. B. D. Sharma University of Health Sciences, Rohtak-124 001, Haryana, India.

Received on : 27.03.2008 Revised : 10.04.09 Accepted : 21.04.09

ABSTRACT

UV-Spectrophotometric method of analysis for zinc insulin in different pH conditions has been standardised. As the absorbance maxima (λ_{max}) for insulin changes in different pH conditions and dilutions, the normal absorbance vs. concentration approach is replaced by *area under absorbance curve* (AUAC) vs. concentration approach. The models or equations (acceptable to FDA) for standard plots in each pH condition are determined instead of relying on conventional, simple, linear relationships. Two curves in each wavelength scan made it essential to carry out curve and range selection. Other simple parameters like inter/intra day variations and precisions are also determined. The relationship between AUAC and concentrations were found to be linear (model 2/ or equation iii) in solution of pH 1.2, buffers of pH 5.8, 7.0; non-linear (model 3/ or equation iv) in buffers of pH 6.0, 6.6 and log based (model 4/ or equation v) in pH 7.5. The concentration range of 0.0-1.0 IU/ml was acceptable statistically (p-level 5.0%) in all pH conditions alongwith *relative standard deviation* (RSD) within 5.0% for all AUAC values. Therefore, UV-spectrophotometric method based on AUAC vs concentration may be used satisfactorily for analysis of zinc insulin.

Keywords: *AUAC; Classic regression; models; peak to valley ratio; absorbance maxima.*

INTRODUCTION

The UV-Visible spectrophotometric methods of analysis are first methods applied on any new chemical drug. The small-scale industry/ academic colleges in developing and underdeveloped countries still rely mainly on this technique. Even in case of protein drugs, UV-absorption methods of analysis are preferable in comparison to various colorimetric methods (Biuret, Bradford, Lowry & Smith's) because of a few number of steps in sample preparation [1]. Whereby this technique is easy to apply, it also suffers some disadvantages like shifting of λ_{max} with dilution and changing pH of the medium [2,3,4]. These changes may or may not be degradation based. In degradation based changes, chromatographic analysis becomes the eventual solution but in non-degradation based changes, still the UV-spectrophotometry remains a favourable analytical technique. Considering zinc insulin as model drug, a case has been represented here in different pH conditions for development of analytical method. Instead of using absorbance, AUAC (area under absorbance curve) has been related to zinc insulin concentrations to get rid of variating λ_{max} in different pH conditions and dilutions. Zinc insulin remains stable for 3 days in 12 N HCl at 37°C, 4-48 days in 0.03N HCl at 105°C and 5 weeks at 40ºC as 2% w/v solution of regular/ neutral insulin [5,6,7]. At pH 7.0, almost all insulin in solution is present as hexamers, at pH 2.0 as dimers and at pH approx. 5.0, an intermediate condition prevails. All these changes do not mean degradation of the insulin[8,9,10]. The minimum stability reported was 1 week at same temperature for lente and ultralente forms. Therefore, the model protein, zinc insulin was considered stable at experimental conditions of 27°3°C and 1.2-7.5 pH range.

To avoid the use of simple, straight line equation i.e. $y_i=\beta+\beta_1 X_i$, the relationship between responses and concentrations have been explained here on basis of polynomic regression ($y_i=\beta+\beta_1 X_i+\beta_2 X_i^2+\beta_3 X_i^3+\beta_4 X_i^4+e_i$) at each pH value.

The overall aim of present study was to develop a UV- spectrophotometric analytical method for zinc insulin in wide range of pH values.

MATERIALS AND METHODS

Materials

Sartorius Electronic Balance LE324S, Hitachi U2800 double beam UV-visible spectrophotometer, Nichipette micro pipettes, Zn Insulin from Novo-Nordisk, Bangalore, India, Potassium dihydrogen orthophosphate ExcelaR from Qualigens Fine Chemicals, Mumbai, India, Sodium hydroxide extra pure, Sodium chloride extra pure and Hydrochloric acid from Loba Chemie Pvt. Ltd., Mumbai, India were used for experiments.

***Correspondence :** dr_gajendra62@yahoo.com

Preparation of Buffers and Insulin Solutions

The different pH solutions (pH 1.2, 5.8, 6.0, 6.6, 7.0 and 7.5) were prepared. All solutions were phosphate buffers except pH 1.2, that was *simulated gastric fluid (SGF)* without enzymes as per instructions given in USP 31/ NF 26 [11]. The pH values of all the solutions were varying within □0.1 as per compendial needs. Six concentrations (0.2, 0.4, 0.6, 0.8, 1.0 and 2.0 IU/ml) of the zinc insulin were prepared in these different pH solutions, with a total of 36 samples per run. Whole experimentation, in each pH condition was completed in 66 runs (11 runs x 6 concentrations), repeated with fresh solutions every time.

Curve Selection

Protein drugs often represent more than one peak in absorbance vs wavelength scans. So there is need for curve selection. The wavelength scans of middle concentration (0.8 IU/ml) were considered insted of scans for all concentrations, in six pH conditions. The UV-visible spectrophotometer was set-up to take readings in absorbance mode, within a wavelength range of 190-300 nm and at scan speed of 400 nm/ min. Thereafter scans were generated from these readings in Microsoft Excel worksheet, version 11, Microsoft office professional 2003. Each scan was result of mean absorbance readings (n=11).

The selection amongst curves (in case more than one curve was obtained) was based on error possibilities determination of two parameters, namely; *area under absorbance curve* (AUAC) and *ends of curves* (baselines). Significantly bigger peak or higher AUAC can have a higher calculated resolution ("baseline resolution"), but the valley can be well above "baseline" so this would introduce error in comparing areas. This limitation was eliminated by *peak to valley ratio* that was chosen as alternate way to assess the effective resolution; that is, how far does the valley go below the peak. A larger ratio implied a better separation and ratio of 1.5 was accepted as limit value for considering curves separated in this case.

However, this was just an attempt to take a complex concept and put it into a simple yes/no format- something the regulators like. Hence, the way drawn was use of three parameters i.e. curve ends, AUAC and *peak to valley ratio* for selection of appropriate curve.

AUAC Determination

Except the curve selection, all other inferences were produced on AUAC basis. Various methods of AUAC determination are available, like trapezoidal, gravimetric, lagrange, cubic spline, parabolas through the origin and log trapezoidal. Purves in his work has compared various methods and rejected lagrange and cubic spline methods because of large variance of their estimates [12]. The simplest method is trapezoidal method, but it overestimates or underestimates the area depending on the concavity of curve, especially when data points are far from each other [13]. In present experimentation, trapezoidal method was used for finding out AUAC. To increase the power of the method, wavelength interval was kept at 1 nm for collection of data points i.e. scan progressed with absorbance recording at each value of wavelength. The calculations were performed by putting step by step formula of area under trapezoid in Microsoft Excel version 11, Microsoft office professional 2003. A general description of calculation has been given in Appendix.

Range Selection

Apart from selecting curves, the *peak resolution* (in form of *peak to valley ratio*) may also provide insight for the range selection. However, this was combined with *adjusted* R^2 values obtained from regression statistics applied on two concentration ranges 0.0-1.0 IU/ml and 0.0-2.0 IU/ml. The range exhibiting least *adjusted* R^2 *value* (i.e. best fitting) and *peak to valley ratio* e"1.5 at each concentration level was finalized. In case, if both ranges appear similar in *peak to valley ratio* e"1.5 and equal *adjusted* R^2 values, the choice can be made on basis of number of significant coefficients obtained from regression statistics. The range explaining relationship with least number of significant coefficients is then finalized. So, regression statistics alongwith *peak to valley ratio* was used for range selection.

Precision

The precision was determined in form of relative standard deviation (RSD) of AUAC values. The RSD values were calculated for AUAC values taken repeatedly (n=11 for solution of pH 1.2 and for all other buffers) at each concentration level.

Selection of Standard Plot Equation (Model Prediction)

In various analytical approaches, the simple linear (y_i=□+□$_1X_i$+e_i) relationship between dependent and independent variable is established and even this is a common practice at commercial level. However, the relationship between dependant (AUAC) and independent (concentration) variables is not always linear. It may be linear or non-linear and thus may introduce a huge systematic error. In view of above, five models (equation ii to vi) of relationships for AUAC vs concentration were compared, starting from polynomic equation (i) and followed by statistical comparison of the estimated coefficeints at 5% p-level [14,15]. Depending upon coefficient values, polynomic equation (i) reduce to one of the five models (ii) to (vi). If the polynomic equation (i) does not reduces to any one of the first three models or equations (ii to iv), the further decision depends on non-linear or log relationships between *y* and *x.* In such cases the valued outcomes are derived from comparing *residual sum of squares* (RSS) values for euations (v) and (vi). The relationship depicting least variation or RSS is then finalized.

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

Model 1 $y_i = \alpha + \beta_1 X_i + e_i$ .....(ii)

Model 2 $y_i = \beta_1 X_i + e_i$ .....(iii)

Model 3 $y_i = \alpha + \beta_1 X_i + \beta_2 X_i^2 + e_i$ .....(iv)

Model 4 $y_i = \alpha X_i^{\beta} e_i$ **or** $\log y_i = \log \alpha + \beta \log X_i + \log e_i$ ...(v)

Model 5 $y_i = \alpha e^{\beta Xi} e_i$ **or** $\log y_i = \log \alpha + \beta X_i + \log e_i$ .....(vi)

In each model, y represents AUAC value, X, α, β are concentration, intercept and coefficients in all equations. The coefficients were determined using polynomic regression in Microsoft Excel version 11, Microsoft office professional 2003.

RESULTS AND DISCUSSION

Curve Selection

Before selecting the satisfactory peak, GMP/GLP status of the spectrophotomer was considered. The accuracy parameters of the spectrohotometer were as under: wavelength within ± 0.2 nm; wavelength reproducibility within ± 0.005 nm; band pass between 1.0-1.5 nm; noise level <0.001 Abs.; baseline stability <0.001 Abs. and baseline flatness within ± 0.0006 Abs.

Wavelength scans (Figs. 1-6) for all concentrations, within 0.2-2.0 IU/ml expressed two curves in all the six pH conditions. The interesting features in Figs. 1-6 are; (i) regular shift in the λ_{max} for both peaks and (ii) astonishingly fixed wavelength values for the valley i.e. 224-225 nm, proving the changing behaviour of λ_{max} with respect to concentration as explained in introduction for protein drugs.

Depending upon qualitative/quantitative approach among Figs. 1-6, we have considered the right curve among the curves in all pH conditions, as only:-

- the right ends of the right curves are flat compared to left ends of the left curves, resuling into more accurate and precise determination of AUAC in case of right curves.
- the right curves are showing more change in AUAC compared to left curves, therefore, determination of AUAC of right curves is more useful compared to AUAC of left curves.
- the left curves are falling in far-UV range while no nascent atmosphere was provided in the sample chamber, hence, making AUAC determination more erroneous in left curves.
- As indicated in Table 1, the ratio of peak to valley is greater for right curves for all concentrations in all pH conditions, suggesting more resolution for right curves.

Table 1. *Peak to valley ratio in different media for different insulin concentrations*

Conc. IU/ml	pH 1.2		pH 6.8		pH 6.0		pH 6.6		pH 7.0		pH 7.6	
	left ratio	right ratio	left ratio	right ratio	left ratio	right ratio	left ratio	right ratio	left ratio	right ratio	left ratio	right ratio
0.2	2.245	2.670	2.758	3.333	2.831	3.482	2.531	3.052	2.581	3.086	2.210	2.600
0.4	1.796	2.184	2.416	2.851	2.436	2.983	2.202	2.637	2.126	2.497	1.913	2.212
0.6	1.489	1.821	2.028	2.367	2.038	2.417	1.891	2.235	1.868	2.172	1.648	1.895
0.8	1.309	1.604	1.736	2.007	1.739	2.041	1.649	1.933	1.612	1.863	1.460	1.670
1.0	1.185	1.445	1.552	1.796	1.546	1.803	1.458	1.699	1.434	1.651	1.331	1.518
2.0	-	-	1.104	1.266	1.101	1.269	1.078	1.239	1.058	1.206	1.031	1.157

** Due to absence of valley, ratio was not calculated at this concentration.*

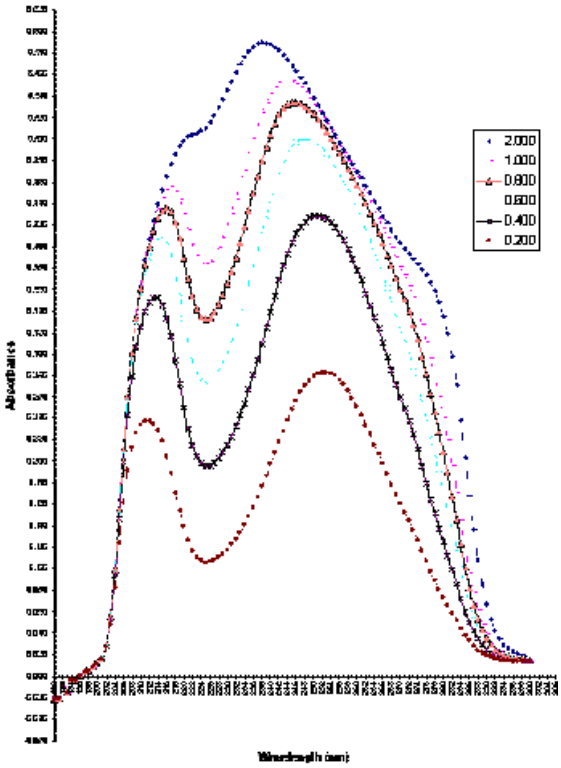

Fig. 1: *Scan of Zinc insulin in solution of pH 1.2.*

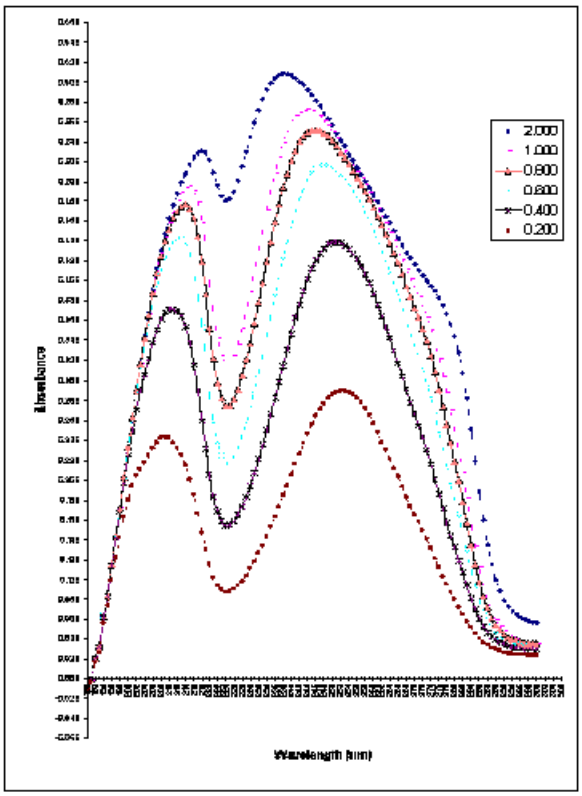

Fig. 2: *Scan of Zinc insulin in buffer of pH 5.8.*

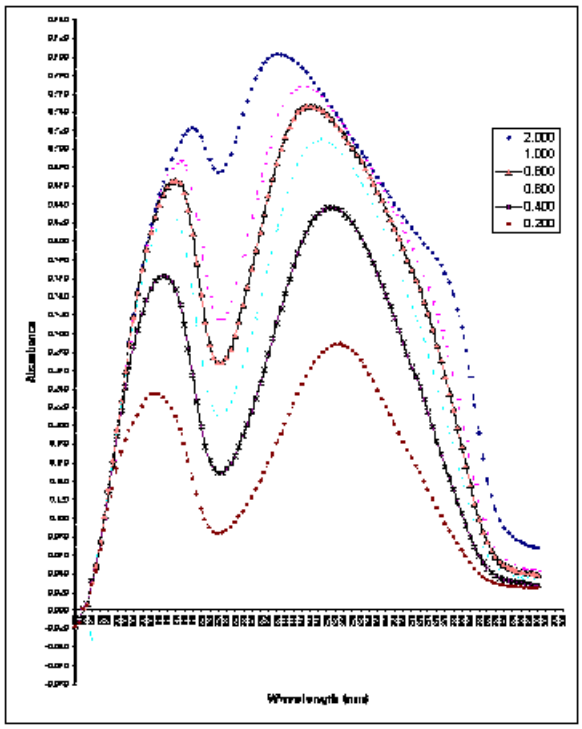

Fig. 3: *Scan of Zinc insulin in buffer of pH 6.0.*

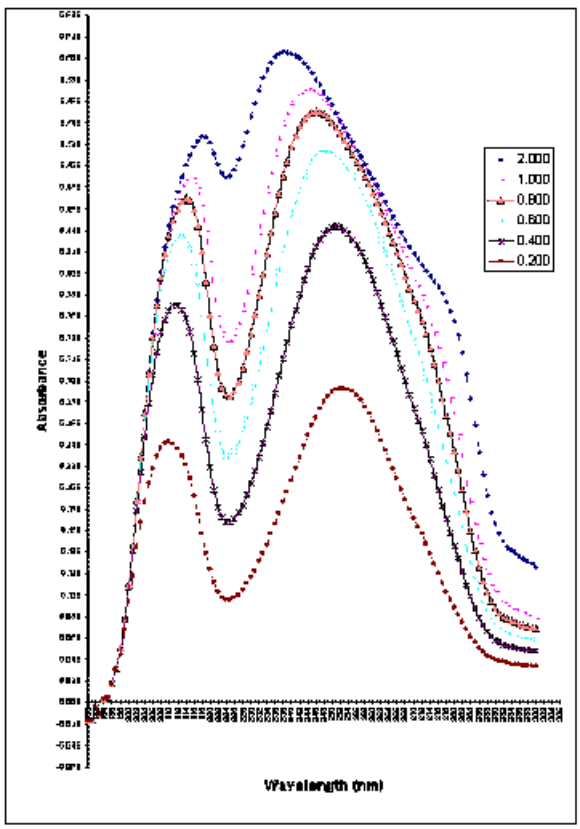

Fig.4: *Scan of Zinc insulin in buffer of pH 6.6.*

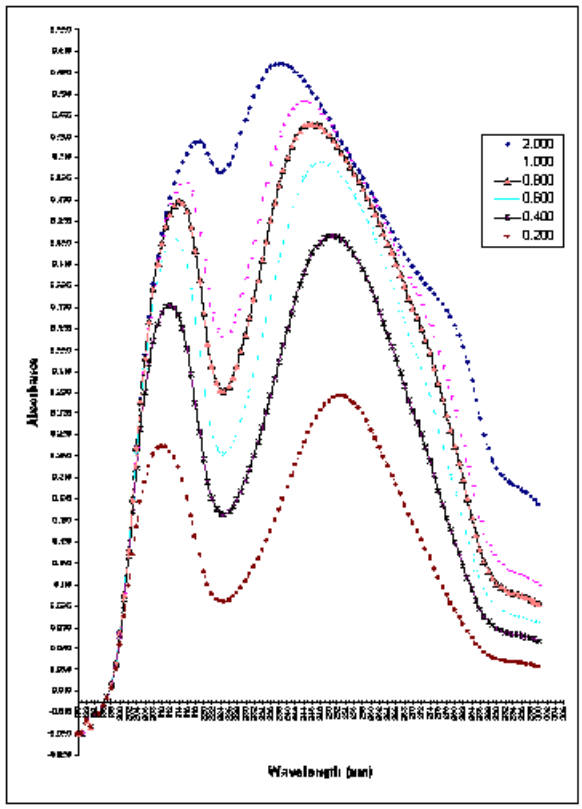

Fig. 5: *Scan of Zinc insulin in buffer of pH 7.0.*

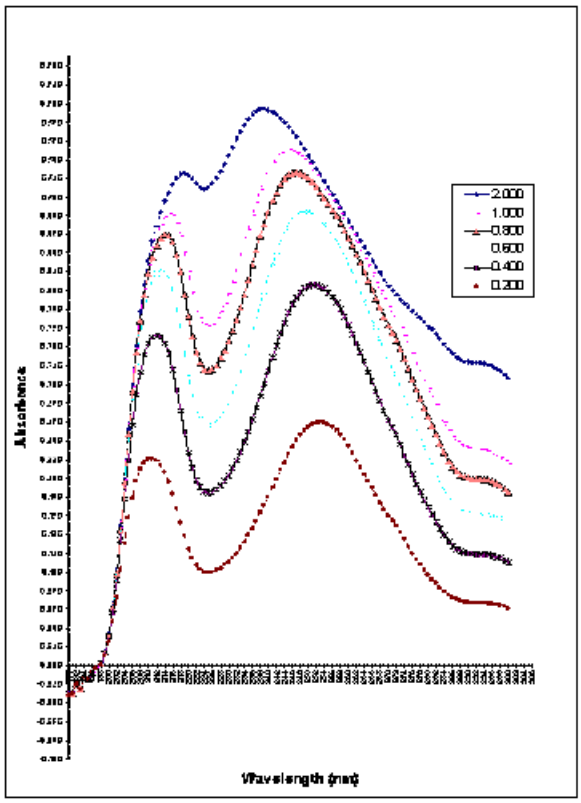

Fig. 6: *Scan of Zinc insulin in buffer of pH 7.5.*

AUAC Determination

The cut off wavelengths for right curves (Figs. 1-6) were found to be lying between 224-225 nm on left ends and 283-292 nm on the right ends (Table 2). Therefore, the mean wavelength selected for AUAC determination was 224.5H"224nm (on left ends) and 287.6H"288 nm (on right ends). The AUAC values were determined from 224-288 nm in all the cases using trapezoidal rule. The calculated AUAC values for concentration range of 0.2-2.0 IU/ml in all pH conditions have been given in Table 3.

Table 2. *Right end wavelengths of right peaks for lowest and highest concentrations.*

Scan No.	pH of solution	Right end wavelengths (nm)		
		0.2 IU/ml	2.0 IU/ml	Average
1	1.20	287 nm	292 nm	289.500 nm
2	5.80	286 nm	290 nm	288.000 nm
3	6.00	286 nm	290 nm	288.000 nm
4	6.60	285 nm	290 nm	287.500 nm
5	7.00	285 nm	289 nm	287.000 nm
6	7.50	283 nm	288 nm	285.500 nm
		Average right end wavelength =		287.583 nm

Table 3. *AUAC values calculated by trapezoidal rule and relative standard deviations (RSD) of calculated AUACs (For right curves)*

	pH 1.2		pH 5.8		pH 6.0		pH 6.6		pH 7.0		pH 7.5	
Conc. (IU/ml)	AUAC (n=11)	RSD	AUAC (n=11)	RSD	AUAC (n=11)	RSD	AUAC (n=11)	RSD	AUAC (n=11)	RSD	AUAC (N=11)	RSD
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.200	11.131	4.946	11.361	3.972	11.250	3.618	11.752	3.847	11.434	4.345	10.920	3.038
0.400	18.011	4.011	18.172	2.571	18.007	2.606	18.750	2.489	18.736	2.489	18.168	2.468
0.600	22.465	3.356	22.759	1.844	22.397	1.933	23.103	1.458	22.913	2.485	22.923	2.534
0.800	25.080	2.937	25.504	1.785	25.218	1.876	25.879	1.546	25.739	1.898	25.980	1.916
1.000	26.820	3.165	27.200	1.215	27.018	1.412	27.719	1.396	27.569	1.346	27.982	1.080
2.000	-*	--	31.068	1.019	30.875	1.154	31.502	1.112	31.398	1.127	31.878	1.001

** Due to absence of valley, AUAC value was not calculated for this concentration.*

Range Selection

As in Table 1, the ratio values are e" 1.5 for all concentrations, except for 2.0 IU/ml and 1.0 IU/ml concentrations in pH 1.2 solution. There is no valley in former concentration but in later, valley exists and ratio is 1.445, that doesn't significantly differe from 1.5. This shows that concentration range of 0.2-1.0 IU/ml is only applicable for analysis withing the pH range of 1.2-7.5. Another quantitative approach for confirming range is polynomic regression statistics, applied on two concentration ranges (0.0-2.0 IU/ml and 0.0-1.0 IU/ml). Considering the *adjusted R^2 values* (Table 4) it is concluded that both ranges are equally efficient in explaining relationship between AUAC and concentrations, but the range 0.0-1.0 IU/ml is resulting into small order equations (depending on coefficient values at p-level of 5%) in all pH conditions, which is more feasible.For range 0.0-2.0 IU/ml, all the coefficients ($\hat{a}_1$ *to* $\hat{a}_4$) are significant in all pH conditions except pH 5.8 whereby $\hat{a}_1$ *to* $\hat{a}_3$ are significant. Therefore, polynomic equation

Table 4. *Statistical parameters of polynomic equation for different concentration ranges of insulin*

Parameters	0.0-2.0 IU/ml	p-value	0.0-1.0 IU/ml	p-value
For pH 1.2				
Adjusted R^2	0.99994		0.99990	
α	0.014	0.879	0.006	0.960
β_1	67.954	0.000	68.688	0.017
β_2	-70.237	0.002	-74.330	0.076
β_3	36.285	0.007	43.114	0.201
β_4	-7.189	0.014	-10.664	0.369
Significance F	3.908E-05		6.702E-03	
Buffer pH 5.8				
Adjusted R^2	0.99975		0.99979	
α	0.036	0.849	0.009	0.960
β_1	68.663	0.001	71.366	0.024
β_2	-70.631	0.007	-85.719	0.096
β_3	36.316	0.028	61.489	0.206
β_4	-7.143	0.059	-19.954	0.302
Significance F	1.671E-04		9.674E-03	
Buffer pH 6.0				
Adjusted R^2	0.99983		0.99996	
α	0.030	0.845	0.004	0.960
β_1	68.869	0.001	71.285	0.011
β_2	-73.205	0.004	-87.696	0.043
β_3	39.843	0.015	64.019	0.093
β_4	-8.276	0.030	-20.579	0.142
Significance F	1.101E-04		4.467E-03	
Buffer pH 6.6				
Adjusted R^2	0.99991		0.99999	
α	0.022	0.846	0.002	0.960
β_1	72.654	0.000	74.650	0.006
β_2	-80.721	0.002	-91.864	0.024
β_3	45.486	0.007	64.056	0.054
β_4	-9.666	0.013	-19.128	0.089
Significance F	5.911E-05		2.503E-03	
Buffer pH 7.0				
Adjusted R^2	0.99992		0.99982	
α	-0.009	0.936	-0.009	0.960
β_1	71.116	0.000	71.112	0.023
β_2	-76.344	0.002	-76.323	0.100
β_3	41.316	0.007	41.280	0.276
β_4	-8.501	0.015	-8.483	0.536
Significance F	5.415E-05		8.876E-03	
For pH 7.5				
Adjusted R^2	1.00000		1.00000	
α	0.005	0.845	0.001	0.960
β_1	65.541	0.000	65.915	0.003
β_2	-60.727	0.000	-62.815	0.013
β_3	28.375	0.001	31.859	0.039
β_4	-5.206	0.002	-6.979	0.088
Significance F	2.391E-06		8.936E-04	

($y_i=\square+\square_1X_i+\square_2X_i^2+\square_3X_i^3+\square_4X_i^4+e_i$) is not reducing into simple equation of less coefficients in any pH condition.

For range 0.0-1.0 IU/ml, only $\hat{a}_1$ is significant in pH 1.2, 5.8, 7.0 conditions; both $\hat{a}_1$ and $\hat{a}_2$ are significant in pH 6.0, 6.6 conditions; and $\hat{a}_1$ *to* $\hat{a}_3$ are significant in pH 7.5 condition. Therefore polynomic equation ($y_i=\square+\square_1X_i+\square_2X_i^2+\square_3X_i^3+\square_4X_i^4+e_i$) in all the conditions is reducing into simple equations ($y_i=\square_1X_i$ in pH 1.2, 5.8, 7.0 and $y_i=\square_1X_i+\square_2X_i^2$ in pH 6.0, 6.6), except pH 7.5 where still three coefficients out of four are remaining in final equation ($y_i=\square_1X_i+\square_2X_i^2+\square_3X_i^3$).

To sum-up, the range 0.0-1.0 IU/ml is finalized, as it explains the AUAC vs. concentrations relationship more simply than 0.0-2.0 IU/ml range.

Precision

As in Table 3, the RSD for AUAC values are within 5.0% in all pH conditions, for all concentrations (0.2-1.0 IU/ml), indicating the precision of data and all inter/intra day variations within 5.0%.

Selection of Standard Plot Equations (Model Prediction)

On the basis of coefficeints (at 5% p-level) in Table 4, the polynomic equation for pH 1.2, 5.8, 7.0 solutions is reducing into model 2 ($y_i=\square_1X_i$) and for pH 6.0, 6.6

conditions it is reducig into model 3 i.e. $y_i=\beta_1 X_i+\beta_2 X_i^2$ _. Thus model 2 (simple linear) and model 3 (quadratic) is finalized for analytical purpose in above mentioned corresponding pH conditions.

The situation is a little complicated for pH 7.5, as final equation is not reducing into any one of the first three models or equations and three out of the four coefficients are having significant values. Therefore, none of the models or equations, out of first three is applicable for describing final relationship between AUAC and concentrations appropriately. Ultimately, the decision depends solely upon RSS values among models 4 and 5. As in Table 5, the RSS values, for log AUAC vs log concentrations (model 4) and log AUAC vs concentrations (Model 5) are less for model 4. Thus it is evident that model 4 is more efficient in explaining relationship between AUAC and concentrations. Thus model 4 has been finalized for analysis purpose in pH 7.5 conditions.

Table 5. *Residual sum of square (RSS) values in pH 7.5 for model 4 and 5.*

	RSS Values	
	0.0-2.0 IU/ ml	**0.0-1.0 IU/ ml**
Model 4	0.01110	0.00194
Model 5	0.05182	0.01386

CONCLUSION

The insulin concentration range 0.0-1.0 IU/ ml can be analyzed using AUAC method. Model 2 is sufficient to explain such relationship in solution of pH 1.2, buffer of pH 5.8 and 7.0 while Model 3 or quadratic equation is good for correlation in buffer of pH 6.0 and 6.6. Model 4 explains the relationship between AUAC and different concentrations in buffer of pH 7.5.

Appendix (Calculation of AUAC by trapezoidal method in excel worksheet)

Step 1: Let cell A1 bear the title wavelength and B1, C1, D1, E1, F1, G1 corresponding to concentration ranges 2.000, 1.000, 0.800, 0.600, 0.400 and 0.200 IU/ml.

Step 2: Insert values 190 to 300 (starting wavelength to end wavelength) in cells A2 to A112 and values of corresponding absorbances in cells B2 to B112, C2 to C112, D2 to D112, E2 to E112, F2 to F112 and G2 to G112 for concentrations 2.000, 1.000, 0.800, 0.600, 0.400 and 0.200 IU/ml.

Step 3: Calculate the first *Area under trapezoid* between 190 and 191 nm wavelengths by putting formula ((B2+B3)/2) x (A3-A2) in cell H3 for concentration 2.000 IU/ml. The area of trapezoid at 2.000 IU/ml concentration in cell H3 can be explained as (Abs. at $ë_{190}$ + Abs. at $ë_{191}$)/2 x ($ë_{191}$– $ë_{190}$).

Step 4: Calculate subsequent *Area under trapezoids* i.e. between 191 and 192 nm, 192 and 193 nm.....299 and 300 nm in corresponding cells H4, H5……

Step 5: Similarly calculate *Area under trapezoids* between 190-300 nm for concentrations 1.000, 0.800, 0.600, 0.400 and 0.200 IU/ml in I, J, K, L and M columns of spreadsheet.

REFERENCES

1 Zaia DAM, et al. Quimica Nova. 1998; 21: 787.

2 Haritoglou C, et al. Invest Ophthalmol. Vis. Sci. 2003; 44: 2722.

3 Romberg RW, Kassner RJ. Biochemistry 1982; 21: 880.

4 Landsman MLJ, et al. J Appl Physiol. 1976; 40: 575.

5 Sanger F, Thompson EOP. Biochem J. 1953; 53: 366.

6 Schultz J, et al. Biochemistry 1962; 1: 694.

7 Brange J. In Galenics of Insulin: The Physico-chemical and Pharmaceutical Aspects of Insulin and Insulin Preparations, Springer-Verlag, 1987, p 59.

8 Blundell TL, Dodson GG, Hodgkin DM, Mercola D. In Advances in protein chemistry. Anfinsen CB, Edsall JT, Richards FM, eds. New York: Academic Press, 1972, p 403.

9 Orci L, et al. Scientific American 1988; 259 (3): 85.

10 Bernardo A, et al. Brazilian J Chem Engg. 2005; 22(03): 331.

11 USP 31st / NF 26th. Rockville, MD, United States Pharmacopeial Convention, Inc., 2007, p 2524.

12 Purves RD. J Pharmacokin Pharmacodyn. 1992; 20: 211.

13 Kwan KC, Yeh KC. J Pharmacokin Pharmacodyn. 1978; 6: 79.

14 Chow SC, Liu JP. In Statistical design and analysis in pharmaceutical sciences., New York: Marcel Dekker, 1995, p 25.

15 Chow SC, Shao J. In: Statistics in Drug Research: Methodologies and Recent Developments., New York: Marcel Dekker, 2002, p 48.