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Preparation and Formulation Optimization of Sugar Crosslinked Gelatin Microspheres of Diclofenac Sodium

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The study was undertaken with an aim to investigate the ability of native sugars (e.g. glucose, fructose and sucrose) to induce crosslinking of gelatin for the preparation of modified release microspheres of diclofenac sodium. The microspheres were prepared by emulsion crosslinking method and they were evaluated for drug content, *in vitro* drug release and size analysis. The results of preliminary trials revealed that the parameters such as drug to gelatin ratio, volume of light liquid paraffin and stirring rate were found to affect the morphology and *in vitro* drug release of microspheres. The microspheres crosslinked with glucose showed highest drug content, good yield and lowest burst effect. The evidence of glucose mediated crosslinking of gelatin was confirmed by DSC. A 3^2 full factorial design was adopted to investigate the joint influence of two variables, amount of glucose (X_1; 1, 1.5 or 2 g) and concentration of gelatin (X_2; 10, 15 or 20% w/v) on the percentage drug released in 60 min (Y_{60}) and the time required for 80% drug dissolution (t_{80}) while keeping the other variables constant. The results of multiple linear regression analysis revealed that for obtaining modified drug release upto 12 h, the microspheres should be prepared using higher level of glucose and middle level of gelatin. Response surface plots are presented to show the effects of X_1 and X_2 on Y_{60} and t_{80}. The drug release pattern fitted well to Korsmeyer and Peppas model indicating anomalous diffusion. An equation was generated by adopting multiple linear regression analysis, for predicting the drug dissolution profile for a check point. Good agreement was observed between the predicted and observed drug dissolution profiles. The results suggest that native glucose could be an interesting agent to crosslink gelatin for obtaining modified release of diclofenac sodium from the microspheres.

Recent research efforts throughout the world have resulted in significant development of novel drug delivery systems. Among these systems, microspheres have emerged as an attractive dosage form due to the advantages it offers like effective taste masking, improvement of flow, safe handling and good sustained drug release properties[1]. Much interest is also being shown these days to the formulations based on the natural polymers owing to its biocompatibility, biodegradability, non-toxicity and ease in availability. In particular, gelatin is extensively used in pharmaceutical industry because of its good film forming properties[2-4]. Gelatin suffers from the main drawback of rapid solubilization in aqueous environment, thus resulting in fast drug release. To overcome this problem, chemical crosslinking agents (i.e. glutaraldehyde[4-6], formaldehyde[7] and glyceraldehyde[8,9]) were used to form partially soluble or insoluble network. However, the safety of using chemical

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crosslinkers is still an open question because of toxic side effects arising from the presence of residual crosslinkers or because of unwanted reactions with drugs resulting in the formation of toxic or inactivated derivatives.

In this respect, alternative methods such as thermal hardening treatment[10] or natural crosslinkers, such as dextran[11] or sugars[12] have been tried for the preparation of gelatin devices. The thermal hardening treatment is time consuming and it is unsuitable for thermolabile drugs. Cortesi *et al.* reported that native or oxidized dextran (polysaccharide) could reduce the solubility of gelatin and sustain the release of model drugs such as TAPP-Br and sodium cromoglycate from gelatin microspheres[13]. In another study, Cortesi *et al.* investigated the crosslinking ability of native and oxidized mono and di saccharides (glucose, fructose and sucrose)[14]. The investigators concluded that although oxidized sugars could be interesting agents to crosslink gelatin, the long-term toxicity cannot be excluded. The authors proposed the possible mechanism involved in gelatin crosslinking by aldose sugars: the aldehyde group of reducing sugars (e.g., glucose or fructose) reacts with the free amino groups of gelatin molecule resulting in the formation of an aminoglycoside. The aminoglycoside further reacts with another amine group of gelatin, originating a crosslinked structure. Despite of the great potential of native sugars as crosslinking agent, only a few articles have been published on this area. It should be pointed out that, no research work is reported that describes the preparation of drug-loaded sugar (mono and di saccharides like glucose, fructose or sucrose) crosslinked gelatin microspheres by adopting systematic formulation approach. The objective of this work was to identify the formulation and processing variables affecting the characteristics of gelatin microspheres crosslinked with sugars. Diclofenac sodium used in the long-term treatment of rheumatoid arthritis, osteoarthritis and ankylosing spondylitis[15] was selected as a model drug. It is rapidly absorbed after oral administration, but common side effects such as gastritis, peptic ulcer and bleeding are observed. Due to short biological half-life (1-2 h), high aqueous solubility and associated adverse effects, it is considered as an ideal candidate for formulating modified release dosage form. In the present study a 3^2 factorial design was used for optimization of diclofenac sodium gelatin microspheres.

MATERIALS AND METHODS

Diclofenac sodium IP and d-glucose were received as gift samples from Torrent Pharmaceuticals and Unicure Pharmaceuticals, India, respectively. Gelatin (JC's Pharmaceuticals, Vadodara), light liquid paraffin (Gujarat Pharmaceuticals, Ahmedabad; viscosity 31 cps), Span 80 (Laser Chemical, Ahmedabad), sucrose (Commercial grade, BDH, Ahmedabad), fructose (Merck Chemicals, Mumbai) and ethyl cellulose (CBH Lab reagents, Ahmedabad) were used as received. The sample of gelatin possessed the following specifications; Bloom strength: 250, isoelectric point: 8.9–9, pH of 1% w/v aqueous solution: 4–5. The solvents (isopropanol and dichloromethane) were of analytical grade.

Method of preparation of gelatin microspheres:

The microspheres were prepared by emulsion crosslinking method. To the aqueous solution of gelatin (15%, 15 ml, 80°), sugar (1.5 g) was added and allowed to react for 5 min. Diclofenac sodium was added to the aqueous solution containing gelatin and sugar. Noticeable color change was not observed in the solution, which indicates absence of 'caramelization' process. The resultant mixture was carefully added with stirring to light liquid paraffin (150 ml, 80°) containing Span 80 (1.5% w/v). The dispersion was stirred using a propeller stirrer (850±25 rpm) to obtain a w/o emulsion. Rapid cooling to 5° using an ice bath hardened the gelatin droplets. After 15 min, isopropanol (15x4 ml, 5°) was added at regular intervals to dehydrate the droplets. Stirring was continued for 2 h, followed by filtration. The microspheres were washed with isopropanol to remove the adhered oil. The microspheres were allowed to dry at ambient conditions (35°, 50% RH). Microspheres of all the batches in the factorial design were coated *in situ* with ethyl cellulose solution (15 ml, 5% w/v ethyl cellulose in dichloromethane containing dibutyl phthalate (10% w/w of the polymer)). The formulation parameters and % yield of the factorial design batches (A1 to A9) are shown in Table1.

Drug content:

The microspheres were evaluated for drug content. Drug loaded microspheres (100 mg) were powdered and suspended in 100 ml phosphate buffer (pH 7.2). The resultant dispersion was exposed to ultrasonic treatment for 4–5 h and filtered through a 0.45 µm membrane filter. The drug content was determined spectrophotometrically (UV/Vis Hitachi U2000 Spectrophotometer, Japan) at 276 nm using a regression equation derived from the standard curve (r^2=0.9978). The drug content of the factorial design batches (A1 to A9) is shown in Table 2.

In vitro dissolution study:

Microspheres equivalent to 100 mg of diclofenac sodium were filled in hard gelatin capsules (white colored, 0

TABLE 1: 3^2 FACTORIAL DESIGN LAYOUT

Batch	Variable levels in coded form		Response values					
	X_1	X_2	Y_{60} (%)	Y_{300} (%)	Y_{480} (%)	t_{80} (min)	Drug content (mg)	Yield (%)
A1	-1	-1	23.62	72.50	89.64	384	25	50
A2	0	-1	24.75	69.93	87.83	396	22	72
A3	1	-1	21.49	61.29	77.11	451	20	74
A4	-1	0	24.88	68.52	88.95	388	24	83
A5	0	0	30.55	65.78	87.97	419	25	69
A6	1	0	23.18	61.91	77.13	452	35	86
A7	-1	1	28.62	75.81	92.79	338	27	64
A8	0	1	27.71	68.68	91.80	379	21	72
A9	1	1	23.34	60.63	80.86	438	20	75
A10	1	-0.5	23.87	55.64	81.16	461	28	78

Coded values	Actual values	
	X_1	X_2
-1	1.0	10
0	1.5	15
1	2.0	20

X_1 = Amount of glucose (g), X_2 = Concentration of gelatin (% w/v), Y_{60}, Y_{300}, Y_{480}= percentage drug released in 60 min, 300 min and 480 min, t_{80} = the time required for 80% drug dissolution, * A10 is the check point batch,

size) and were evaluated for *in vitro* dissolution study. The study was carried out in accordance with the USP 24 Type I rotating basket apparatus (Electrolab, model TDT–06T, Mumbai) using 900 ml phosphate buffer (pH 7.2, 37±0.5°) at 50 rpm. A muslin cloth (200#) was tied over the basket to prevent the slippage of microspheres from the basket. Sample (5 ml) were withdrawn at regular time intervals,

TABLE 2: EFFECT OF TYPES OF SUGAR ON CHARACTERISTICS OF MICROSPHERES

Parameters	Batch F1 (Glucose)	Batch F2 (Sucrose)	Batch F3 (Fructose)
Drug content (mg)	35	18	15
Yield (%)	86.00	69.56	57.25
Geometric mean diameter (μm)	25.40	27.25	29.48
Y_{60} (%)	23.18	56.06	48.47
t_{80} (min)	452	401	249

Y_{60}=percentage drug released in 60 min, t_{80} = the time required for 80% drug dissolution

filtered through a 0.45 μm membrane filter, diluted suitably and analyzed spectrophotometrically at 276 nm. An equal amount of fresh dissolution medium (37±0.5°) was replaced immediately after withdrawal of the test sample. The percentage drug dissolved at different time intervals was calculated. The study was performed in triplicate for each batch. The time required for 80% drug release (t_{80}) was calculated using Korsmeyer and Peppas model[16].

Morphology and size analysis of microspheres:

The morphology of gelatin microspheres was evaluated by Scanning Electron Microscopy (Philips, XL-30, ESEMTMP). The microsphere size distribution was determined by the simple microscopy method using a calibrated stage micrometer. Geometric mean diameter (μm) was calculated by using the Eqn. 1, $Xg = 10x((n_i x \log X_i)/N)..(1)$, where, Xg is the geometric mean diameter, n_i is the number of particles in the range, X_i is mid–point of the range and N is the total number of particles (N=100).

Differential scanning calorimetry:

The cross-linking of gelatin with glucose was characterized by using a differential scanning calorimeter (Perkin-

Elmer DSC-2), attached to a thermal analyzer. Samples were scanned in aluminum pans, under static air atmosphere, at a heating rate of 10° min^{-1} in the temperature range 40-240°.

Experimental design:

A statistical model (Eqn. 2) incorporating interactive and polynomial terms was used to evaluate the response, $Y=b_0+b_1X_1+b_2X_2+b_{12}X_1X_2+b_{11}X_{11}+b_{22}X_{22}....(2)$, where Y is the dependent variable, b_0 is the arithmetic mean response of nine runs and b_i is the estimated coefficient for the factor X_i. In the present investigation, the amount of sugar and concentration of gelatin were selected as independent variables, whereas Y_{60} and t_{80} were chosen as dependent variables. The other variables such as volume of light liquid paraffin, stirring speed and drug to gelatin ratio were kept constant.

RESULTS AND DISCUSSION

The presence of 1.5% Span 80 (HLB 6–7) was found to be essential to minimize aggregation of gelatin droplets during the emulsification step. The type and quantity of the oil phase played an important role in controlling the diameter of microspheres. Two types of liquid paraffin (heavy or light) were tried. When heavy liquid paraffin was used, greater resistance was offered in the process of dispersion leading to the formation of aggregated microspheres whereas discrete microspheres were obtained when light liquid paraffin was used. Thus, light liquid paraffin was selected and effect of its volume (100, 150 and 200 ml) on the characteristics of microspheres was studied. Discrete microspheres were obtained when 150 ml of light liquid paraffin oil was used. To study the effect of stirring speed, microspheres were prepared at 250, 550 and 850 rpm. At 850 rpm, microspheres with higher yield and good sphericity were obtained. For preliminary screening, three different drug to gelatin ratios (0.5:1, 1:1 and 1.5:1 w/w) were tried. The formulation prepared using 1:1 w/w drug to gelatin ratio showed highest t_{80} (287 min), drug content (32 mg) and yield (69%). However, the microspheres showed a burst effect (40% drug release in 1 h) and a relatively faster drug release thereafter (total drug release in 6 h). *In situ* coating of microspheres with ethyl cellulose significantly reduced the burst effect (23% drug release in 1 h).

A study was also carried out to evaluate the effect of different types of sugar as crosslinking agent. The comparison of different parameters is depicted in Table 2. The microspheres prepared using glucose (Batch F1) showed highest drug content, yield and lowest burst effect (Y_{60}). Hence, glucose was selected for further studies in the experimental design.

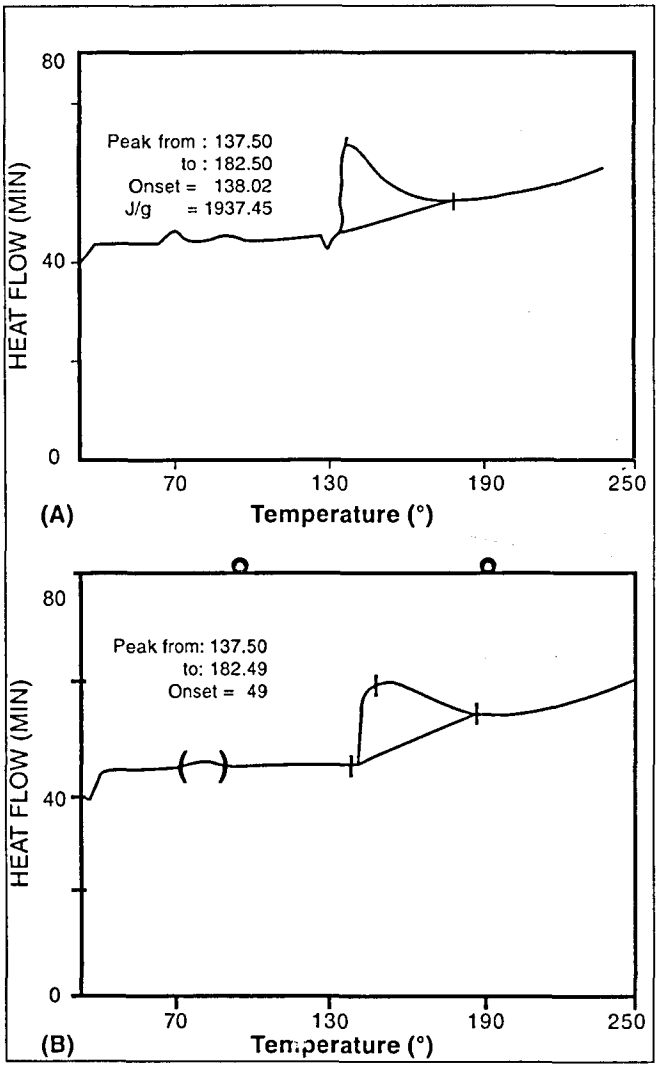

Fig. 1: DSC of the Samples

(A) DSC of pure gelatin, (B) DSC of the glucose crosslinked gelatin microspheres of best batch A6.

The thermal properties of crosslinked gelatin microspheres were analysed by using differential scanning calorimeter attached to a thermal analyzer. The glucose-mediated crosslinking was evidenced by a variable shift of glass transition temperature (Tg) from peak at 141.1 to 146.6° as shown in fig.1.

The compositions of the nine batches of the factorial design are shown in Table 2. The polynomial equations re-

lating the responses Y_{60} and t_{80} to the transformed factors are shown here: $Y_{60}=28.52-1.52X_1+1.64X_2-0.79X_1X_2-3.48X_1^2-1.28X_2^2$...(3), ($r=0.9212$, DF=8, $F=3.36$), $t_{80}=412.65+38.57X_1-12.52X_2+8.22X_1X_2+10.23X_1^2-21.69X_2^2$...(4), ($r=0.9886$, DF=8, $F=25.79$)

The main effects (X_1 and X_2) represent the average result of changing one factor at a time from its low to high value. The interactions (X_1X_2) show how the response value changes when the two factors are simultaneously changed. The polynomial terms (X_1^2andX_2^2) were included in the model to investigate non-linearity. The derived polynomial equations can be used to draw conclusions after considering the magnitude of coefficient and the mathematical sign it carries (i.e. positive or negative).

The response values for the nine batches showed a wide variation; Y_{60} ranged from 21.5 to 30.6% and t_{80} ranged from 338 to 452 min. The data clearly indicates that Y_{60} and t_{80} values depend significantly on the selected independent variables. The high value of correlation coefficient ($r=0.9212$ for Y_{60} and $r=0.9886$ for t_{80}) indicates a good fit. Figs. 2 and 3 shows the response surface plots drawn using SigmaPlot® software (version 3.0).

The Eqn. 3 represents that the coefficients associated with X_1 carries a negative sign. The Y_{60} was found to be inversely related to the amount of sugar since the degree of cross-linking is dependant on the quantity of sugar. Greater the degree of crosslinking, slower will be the drug dissolution. Faster drug release is expected from water-soluble uncross-linked gelatin. As shown in fig. 2, it is evident that high level of X_1 and low level of X_2 appear to control the burst effect. One can obtain the desired value of Y_{60} by properly choosing the levels of X_1 and X_2. At high level of sugar, a higher degree of cross-linking is achieved which forms a denser insoluble network, thus reducing the burst effect. At low levels of gelatin, the number of molecules of gelatin that participates in crosslinking reaction will be more as compared to that when high level of gelatin is used.

The Eqn. 4 reveals that the effect of factor X_1 is greater in magnitude than that of factor X_2. It may be concluded from fig. 3 that, when high level of sugar is used, crosslinking reaction is favored resulting in a slower drug release. Intermediate concentration of gelatin (15%) is preferred because at higher concentration (20%) an increase in viscosity of gelatin solution was observed. This in turn offers a high resistance to the shearing action of the emulsion which

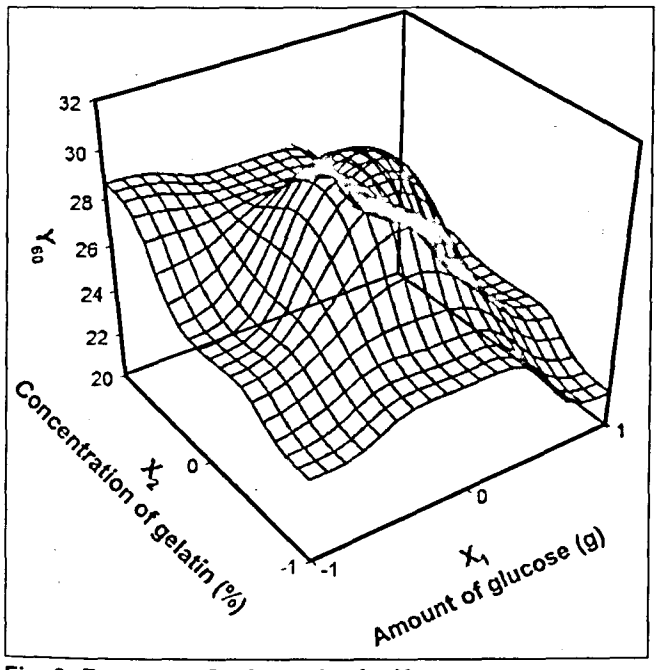

Fig. 2: Response Surface plot for Y_{60}

Response surface plot for Y_{60}, X_1 = Amount of glucose (g), Concentration of gelatin (% w/v), Y_{60} = percentage drug released in 60 min

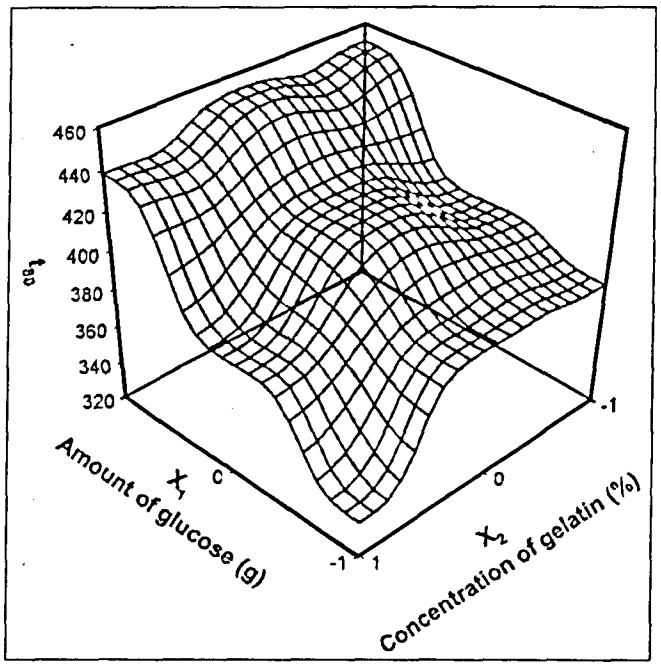

Fig. 3: Response Surface plot for t_{80}

Response surface plot for t_{80}, X_1 = Amount of glucose (g), X_2 = Concentration of gelatin (% w/v), t_{80} = the time required for 80% drug dissolution

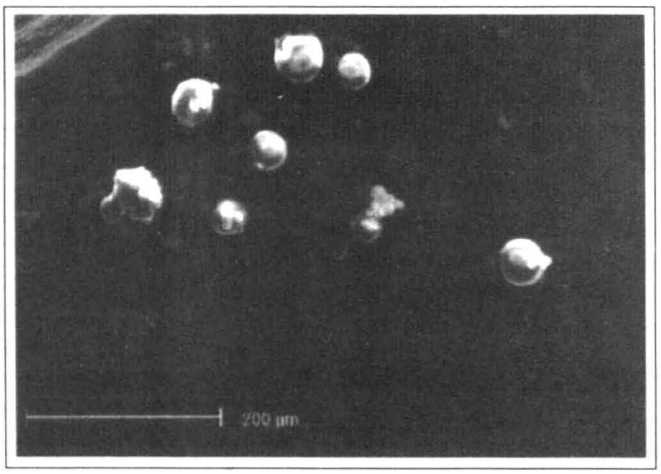

Fig. 4: Scanning electron micrograph (SEM) of gelatin microspheres of the best batch A6.

then causes a concomitant increase in the particle size of microspheres (geometric mean diameter=27.2 µm) and decrease in t_{80} (438 min).

An ideal modified formulation is one from which a loading dose (<30%) is released in the first hour and the remaining 70% of the drug is released thereafter at a constant rate (6.36% per hour). The following constraints were chosen for the selection of the best batch; Y_{60}<30%, 52%<Y_{300}< 62% and 70%<Y_{480}<80%. The batches A3 and A6 met all the three selection criteria. For microspheres, drug content is an important parameter because it determines the quantity of microspheres required to deliver the dose of a drug. The microspheres of batch A6 exhibited higher drug content (35 mg) and yield (86%). The microspheres showed good spherical geometry with little aggregation as shown in the scanning electron micrograph (fig. 4). Differential scanning calorimetry technique was used to characterize the thermal behaviour of the sugar cross-linked gelatin microspheres. Usually, time-dependent thermal relaxation of a polymer is evidenced by an endothermic glass transition temperature (Tg). The determination of the Tg of gelatin microspheres obtained before and after treatment with sugars was performed. The presence of a sugar-mediated cross-linking is evidenced by a variable shift of Tg as evident in fig. 1. Thus, we can confirm that cross-linking of gelatin occurred. Thus, batch A6 was ranked as a potential candidate for obtaining the required drug release. The drug content and yield of all the nine batches is shown in Table 2.

The method of Bamba *et al.* was adopted to decide the most appropriate model[17]. For the best batch (Batch A6), the release profile fitted best to Higuchi model[18] (F=35.21) giving the least residual sum of squares as compared to Korsmeyer and Peppas model (F=35.35) and zero–order model (F=70.20). This superiority is, however statistically insignificant as shown by F–ratio test. For the batch A6, the values of correlation coefficient were found to be 0.9830, 0.9804 and 0.9605 for Korsmeyer and Peppas, Higuchi and zero–order models, respectively. Out of the nine batches, Korsmeyer and Peppas model fitted well to 5 batches whereas the Higuchi model showed good fit in 4 batches. Thus, Korsmeyer and Peppas model was selected. For the Korsmeyer and Peppas model, the values of slope and intercept were found to be 0.5824 and –1.6432 respectively. From the value of slope (n=0.5824, 0.45<n<0.89), it can be concluded that the drug is released by diffusion of anomalous type (non–Fickian).

Peck *et al.* derived a mathematical relationship for the expression of complete dissolution profile[19]. An effort was made in this study to derive a similar type of relationship. A linear interactive model was generated using the data of percentage drug released from all the nine batches. The Korsmeyer and Peppas model showed good fit to the data set and hence log time was chosen as an additional independent variable. The multiple linear regression analysis was performed using the actual values. The derived equation describing the dissolution pattern is shown here: $Y=-100.76+18.41X_1-0.31X_2-0.51X_1X_2-6.33X_1^2+0.05X_2^2+65.15 \log t$...(5), (r=0.9834, DF=71, F=318.2) where, Y is the percentage drug dissolved at time t. The high value of r indicates a good fit. The F–test was found to be significant at

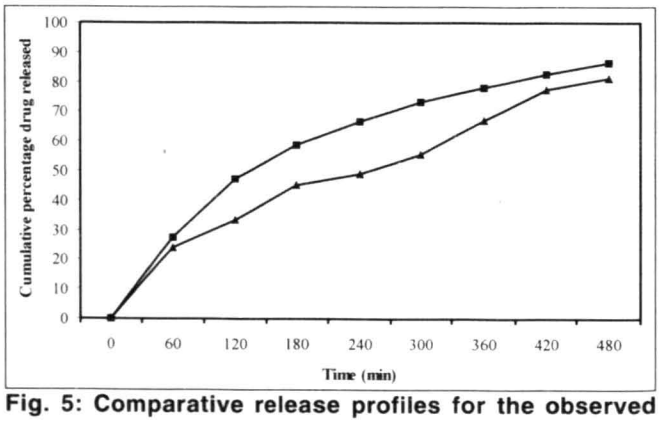

Fig. 5: Comparative release profiles for the observed and predicted check point

Comparative *in vitro* release profiles for the observed and predicted check point, (-■-) release profile of predicted check point, (-▲-) release profile of observed check point

$p<0.05$. The derived equation may be used for calculating percentage drug release at different time interval from the batches within the factor space.

A checkpoint batch (A10) was prepared, close to the settings of batch A6, to validate the derived equation. The predicted and observed release profiles for the check point are depicted in fig. 5. The experimental release profile compared quite well with the predicted release profile obtained from the mathematical model. The predicted t_{80} values for the check point was found to be 458 min, which is in good agreement with the observed values (t_{80}=461 min).

The results reveal that gelatin microspheres containing diclofenac sodium could be successfully prepared using native glucose as a crosslinking agent. The parameters such as amount of sugar, concentration of gelatin, drug to gelatin ratio, volume of light liquid paraffin and stirring speed should be critically controlled. Sugar cross-linked gelatin microspheres were able to sustain the release of diclofenac sodium. It may be noted that at higher level of glucose and lower level of gelatin, highest t_{80} was obtained. Sugar crosslinking can be conceded as safer methods, which obviate the toxicity problems associated with the use of chemical crosslinkers. Finally it is concluded that the required product characteristics can be obtained by systematic approach to the formulation development work.

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