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Quality by design approach to understand the process of nanosuspension preparation

Sudhir Verma[a], Yan Lan[b], Rajeev Gokhale[b], Diane J. Burgess[a,*]

[a] Department of Pharmaceutical Sciences, University of Connecticut, 69 North Eagleville Road, Storrs, CT 06269, USA
[b] Abbott Laboratories, Abbott Park, IL 60064, USA

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ABSTRACT

Quality by design (QbD) principles were explored to maximize the understanding of the unit operation of microfluidization, for the preparation of nanosuspensions using indomethacin as a model drug. The effects of key formulation and process variables (drug concentration, stabilizer type, stabilizer concentration, temperature, milling time and microfluidization pressure) were investigated by executing a $2^{(5-1)}$ factorial design. Particle size, zeta potential and the physical form of the drug constituted the critical quality attributes (CQAs). Multiple linear regression analysis and ANOVA were employed to identify and estimate the effect of important parameters, establish their relationship with CQAs, create design space and model the process of microfluidization for predictive purposes. In order of importance, milling time, microfluidization pressure, stabilizer type, temperature and stabilizer concentration were identified as critical parameters affecting the formation and stability of nanosuspensions. Interaction between homogenization pressure, temperature and milling time also significantly affected the nanosuspension particle size. No correlation was found between the zeta potential and the storage stability. No change in the physical form of indomethacin was observed on storage for 28 days at 4 °C and 25 °C. This research highlights the level of understanding that can be accomplished through a well designed study based on the philosophy of QbD.

1. Introduction

Quality by design (QbD) emphasizes systematic development of pharmaceutical products based on sound scientific principles. QbD aims at making the regulatory approval process more flexible without compromising patient safety. Regulatory agencies such as the US FDA, have championed QbD principles to ensure rapid availability of high quality pharmaceutical products (Wechsler, 2008). QbD encompasses the application of tools such as: critical quality attributes (CQAs); design of experiment (DOE); risk assessment; and process analytical technology (PAT) to the development of pharmaceuticals. QbD stresses the need to thoroughly understand critical product (material) and process parameters with the aim of achieving successful product development with predefined quality attributes (Lionberger et al., 2008). Accordingly, quality is built into the product and is not merely established by testing the end product (Yu, 2008; FDA May 2006). Critical quality attributes are properties that need to be controlled as they impact either patient safety or efficacy. In addition, there could be other parameters that affect business attributes such as yield and cycle time. An in-depth knowledge of the effects of material properties and manufacturing processes on critical product attributes and hence on product performance assists the research scientist in creating a design space for the product.

Design space as defined by the FDA (FDA May 2006) is "The multidimensional combination and interaction of input variables (*e.g.*, material attributes) and process parameters that have been demonstrated to provide assurance of product quality." A design space (DS) is a multidimensional region with respect to the process parameters within which there is a high assurance that the CQAs remain within specifications for the shelf life of the product. A DS can be created for each unit operation or for a process as whole. Moreover, DS provides the flexibility of operating within that space without further regulatory approvals. Additionally, DS is produced through a well organized set of experiments known as design of experiments. This tool offers efficient means to simultaneously test for variable effects and interactions and relates causative relationships between process parameters, input materials and quality attributes. DOE helps in identification and classification (critical or non-critical) of various formulation and process parameters affecting product quality. Interactions between various input variables can be detected and quantified by a well implemented DOE. It also affords predictive capabilities of the desired quality attributes over the design space (Lionberger et al., 2008). With a comprehensive knowledge of all the

* Corresponding author. Tel.: +1 860 486 3760; fax: +1 860 486 0538.
E-mail address: d.burgess@uconn.edu (D.J. Burgess).

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Table 1
Marketed nanosuspension based products (Rabinow, 2004; Wagner et al., 2006).

Drug	Indication	Route	Marketed	Company
Rapamune® (sirolimus)	Immuno-suppresant	Oral	2001	Wyeth/Elan
Emend® (aprepitant)	Anti-emetic	Oral	2003	Merck/Elan
Tricor® (fenofibrate)	Lipid regulation	Oral	2004	Abbott/Elan
Megace ES® (megesterol acetate)	Eating disorders	Oral	2005	Par/Elan
Triglide® (fenofibrate)	Lipid regulation	Oral	2005	Sciele Pharma/Skyepharma
Abraxane® (paclitaxel)	Anti-cancer	I.V.	2005	Abraxis Bioscience/Astrazeneca

sources of variability in a process, PAT tools can then be employed to control these processes in the established design space. Such extensively studied processes are considered by the FDA as well understood processes (Alfnan, 2004) and this forms the core of the QBD initiative. Accordingly, it is important to consider QbD when developing new pharmaceutical formulations and processes. We have applied a QbD approach to investigate formulation development and process characterization of nanosuspensions.

In the recent years nanosuspensions have emerged as one of most promising dosage forms for the formulation of water insoluble drugs (Müller et al., 2001; Patravale et al., 2004). Nanosuspensions can be defined as sub-micron colloidal dispersions of discrete drug particles, which are stabilized with the help of polymers, surfactants or a mixture of both. The small size of particles in nanosuspensions offers a large drug surface area and increase the dissolution rate of poorly soluble drugs. This translates into improved bioavailability, rapid onset of action, reduced food effect and other desirable biopharmaceutical effects that ensure superior clinical performance of BCS Class II and IV compounds (Rabinow, 2004; Kesisoglou et al., 2007). Nanosuspensions are influencing every stage in the drug development process from preclinical, where they are being used to formulate compounds for toxicological studies, to the final marketed dosage forms (Kesisoglou et al., 2007). As many as six nanosuspension based formulations have been marketed in the last eight years (Table 1). No harsh chemicals or co-solvents are used in the formulation of nanosuspensions. This, along with other benefits such as simplicity of formulation, high drug loading capability and ease of scale up, are responsible for the ready acceptance of nanosuspensions by the industry.

Nanosuspensions can be prepared by two processes: (i) top-down and (ii) bottom-up. Top-down processes involve particle size reduction of large drug particles into smaller particles using various wet milling techniques such as media milling, microfluidization and high pressure homogenization. In the bottom-up approach the drug is dissolved in an organic solvent and is then precipitated by adding an anti-solvent in the presence of a stabilizer. Various adaptations of this approach include: (i) solvent-antisolvent method (ii) supercritical fluid processes (iii) spray drying and (iv) emulsion–solvent evaporation. Though bottom-up approaches have shown promise, considerable gaps exist and more work is required before this technique can be commercialized. On the other hand the top-down approach, especially media milling, has been readily accepted by the industry and in fact most of the nanosuspension products currently available on the market are prepared using this technique.

Media milling involves the use of various milling media such as zirconia, and glass balls/beads to reduce the particle size of the compounds and produce sub-micron particle dispersions. One of the main disadvantages of this technique is possible contamination of the product by the particles of the milling media due to erosion during processing. To overcome this problem Elan Drug Delivery Systems have developed and patented highly cross-linked polystyrene beads which were used as milling media to produce Nanocrystals® (Merisko-Liversidge et al., 2003). Microfluidization is another milling technique which results in minimal product contamination (Illig et al., 1996), however, this technique has not yet been explored extensively. Besides minimal contamination, this technique can be easily scaled up (Illig et al., 1996). In this method a sample dispersion containing large particles is made to pass through specially designed interaction chambers at high pressure. The specialized geometry of the chambers along with the high pressure causes the liquid stream to reach extremely high velocities and these streams then impinge against each other and against the walls of the chamber resulting in particle size reduction. The shear forces developed at high velocities due to attrition of particles against one another and against the chamber walls, as well as the cavitation fields generated inside the chamber are the main mechanisms of particle size reduction with this technique (Gruverman, 2003). Literature data is available in the fields of food and pharmaceutical sciences that involve the use of microfluidization processes, however, most of these are limited to the preparation of emulsions, liposomes and microcapsules. There are only a few publications in the public domain which deal with the use of microfluidization for the production of sub-micron pharmaceutical suspensions (Illig et al., 1996). Many of these deal with the preparation of sustained release polymeric nanoparticles (Bodmeier and Chen, 1990; Kwon et al., 2002) and not with immediate release crystalline drug nanosuspensions.

The purpose of this study is to evaluate the process of microfluidization for the preparation of nanosuspensions in order to identify and estimate various critical process and formulation parameters. Another aspect of this study is to use the principles of quality by design along with appropriate design of experiments to obtain a comprehensive knowledge about the process of particle size reduction as it applies to microfluidization. Data analysis using ANOVA and multifactor analysis is performed to: assist in elucidating interactions between different variables; rank order the various process and formulation variables; and help provide a predictive model for the process. This study also reports the physical stability of the nanosuspensions at 4 °C and 25 °C as a function of processing and formulation conditions. To the best of our knowledge, there has been no previous detailed publication related to the concepts of quality by design for the production of nanosuspensions via microfluidization. Besides providing an in-depth knowledge on the microfluidization milling process this study also underscores the valuable information that can be gained by applying QbD principles.

2. Materials and methods

2.1. Materials

Indomethacin USP, 1-(p-chlorobenzoyl)-5-methoxy-2-methyl-indole-3-acetic acid, γ polymorph, was purchased from PCCA (Houston, TX). Methocel (hydroxypropyl methylcellulose) E5 Premium LV (HPMC E5) and Dowfax 2A1 (alkyldiphenyloxide disulfonate) were generously gifted by Dow Chemical Company (Midland, MI). Glycerin USP was purchased from PCCA (Houston, TX). Acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ), dibasic sodium phosphate and monobasic sodium phosphate was obtained from Sigma–Aldrich (St. Louis, MO).

Table 2
Experimental protocol.

Run order	Indomethacin (parts)	Stabilizer type	Stabilizer concentration (parts)	Processing temperature (°C)	Homogenization pressure (psi)
1	7	Dowfax 2A1	3	25	18,000
2	7	Dowfax 2A1	3	5	10,000
3	5	HPMC E5	2	15	14,000
4	3	Dowfax 2A1	1	5	10,000
5	3	HPMC E5	1	5	18,000
6	7	HPMC E5	3	25	10,000
7	5	Dowfax 2A1	2	15	14,000
8	7	Dowfax 2A1	1	5	18,000
9	3	HPMC E5	1	25	10,000
10	3	Dowfax 2A1	1	25	18,000
11	5	HPMC E5	2	15	14,000
12	7	HPMC E5	1	25	18,000
13	3	Dowfax 2A1	3	25	10,000
14	5	Dowfax 2A1	2	15	14,000
15	3	HPMC E5	3	25	18,000
16	5	Dowfax 2A1	2	15	14,000
17	3	HPMC E5	3	5	10,000
18	5	HPMC E5	2	15	14,000
19	3	Dowfax 2A1	3	5	18,000
20	7	Dowfax 2A1	1	25	10,000
21	7	HPMC E5	3	5	18,000
22	7	HPMC E5	1	5	10,000

2.2. Preparation of nanosuspensions

The required amount of indomethacin was dispersed in 100 ml of the stabilizer solution using a mechanical stirrer to form a macro-suspension of the drug. The macro-suspension was homogenized at 10,000 rpm for 10 min using a PowerGen 700 D (Fisher Scientific) lab homogenizer to break up any lumps of the drug that may be present in the macro-suspension. Particle size reduction was carried out by processing this pre-conditioned macro-suspension through a microfluidizer model 110Y (Microfluidics, Newton, MA) at a desired pressure for 90 min. The bulk temperature of the nanosuspension was maintained within ±1 °C during processing at the desired value by using a circulating water bath (Grant Ltd. 6, Grant Instruments, Cambridge, UK).

2.3. Experimental design

It is important to review historical data to ascertain factor and response selection. This could consist of information obtained from previous commercialized products and processes. Some possible sources of useful information include: (a) deviation data such as factors not meeting their proven acceptable ranges or operating ranges or responses not meeting their control limits or specifications, (b) exploratory lab trial data, (c) analytical data, (d) equipment capability and manufacturer's specifications including operational and performance qualifications. Such historical information combined with experience of previously used excipients on formulation, selection of solvents, equipment sets and analytical methods can constitute a platform for experimental design.

Preliminary experiments were conducted (data not shown) and it was determined that the drug concentration, the type of stabilizer, and the concentration of the stabilizer are important formulation variables that can significantly affect the formation of stable nanosuspensions. It was also confirmed that the operating parameters are achievable. It was established that the bulk temperature of the suspension during processing, the total time of milling and the microfluidization pressure are critical processing variables which affect particle size reduction via microfluidization (Illig et al., 1996; Kesisoglou et al., 2007). A systematic approach was used to evaluate the effect of various formulation and process variables on the formation and stability of nanosuspensions. A half, five factorial design $2^{(5-1)}$ was employed to investigate the effects of various parameters on the nanosuspension characteristics. The five independent variables and the two levels studied in this investigation were concentration of indomethacin (7 parts/3 parts), stabilizer type (HPMC E5/Dowfax 2A1), stabilizer concentration (3 parts/1 part), microfluidization pressure (18,000 psi/10,000 psi) and processing temperature (25 °C/5 °C). The total time of microfluidization was maintained at 90 min but samples were also withdrawn at 15 min, 30 min, and 60 min to study the kinetics of particle size reduction. Seven parts of indomethacin were equivalent to 0.5% (w/v) concentration of the drug and all other parts were calculated accordingly. The experimental design was created using Minitab software (version 15, Minitab Inc.) and is shown in Table 2. Six center points were also added to the design to incorporate non-linearity into the responses. Center points were added to make the design more robust and enhance the predictive power of the model based on the design. To reduce systematic errors experiments were randomized completely.

2.4. Physical stability of nanosuspensions

Prepared nanosuspensions were divided into two parts and kept at 4 °C and 25 °C for four weeks. Samples were withdrawn after one week, two weeks and four weeks. The samples were characterized for particle size, zeta potential and physical form of the drug.

2.5. Response measurements

The responses observed were particle size distribution (mean, D50 and D90), zeta potential and physical form of the drug using XRD. Complete details of the measured responses at different time points are given in the Table 3.

2.6. Characterization of nanosuspensions

2.6.1. Particle size analysis

The volume weighted particle size distribution of the nanosuspensions was determined by dynamic light scattering using Submicron Particle Sizer Autodilute Model 370 (Nicomp Particle Sizing Systems, Santa Barbara, CA) at 25 °C. Samples were diluted with 30% glycerin (including stabilizer to match the stabilizer concentration to that of the nanosuspensions and was pre-saturated with indomethacin) before measuring particle size. Viscosities of

Table 3
Response variables investigated following processing and storage.

Particle size	Zeta potential	XRD
Mean, D50 and D90 at 15 min, 30 min, 60 min and 90 min of processing	90 min of processing	90 min of processing
Mean, D50 and D90 at one week, 4 °C and 25 °C	One week	One week, 4 °C and 25 °C
Mean, D50 and D90 at two weeks, 4 °C and 25 °C	Two weeks	Two weeks, 4 °C and 25 °C
Mean, D50 and D90 at four weeks, 4 °C and 25 °C	Four weeks	Four weeks, 4 °C and 25 °C

the diluted samples were measured using a Brookefield viscometer (Model DV III, Stoughton, MA) and were incorporated in the particle size calculations. Three dilutions for each sample were prepared and their average and standard deviations are reported. Sample run time was approximately 5 min.

2.6.2. Zeta potential measurement

The zeta potential values of the nanosuspensions were determined using a Zeta Plus (Brookhaven Instruments Corporation, Holtsville NY). Nanopure water (Ultrapure water systems, Barnstead) (with constant conductivity of 18.2 MΩ cm) was used for the preparation of all dilutions. Samples were diluted in a similar fashion to that described above for the particle size distribution. All measurements were made in triplicate and the mean values and standard deviations are reported.

2.6.3. Solid state characterization

Eight milliliter of the suspension was centrifuged at 12,000 rpm (rcf: $9659 \times g$) using a Minispin centrifuge (Eppendorf, Westbury, NY) for 10 min to separate the solids. X-ray diffraction patterns were obtained using an X-ray diffractometer (Model D5005, Bruker AXS Inc., Madison, WI) using Cu-kα radiation, a voltage of 40 kV, and a current of 40 mA. The scanning rate was 5°/min over a 2θ range of 5–50° with a sampling interval of 0.01°.

2.6.4. Solubility measurements

Excess of indomethacin was stirred with the stabilizer solution (0.5% w/v) at 25 °C for 24 h. The suspension was then filtered through 0.1 μm PVDF filter and the amount of indomethacin dissolved was analyzed by HPLC. Three samples were analyzed for each stabilizer solution.

2.6.5. HPLC analysis

The amount of indomethacin dissolved in the stabilizer solution was quantified using a C-18 Zorbax® column and a mobile phase which was a mixture of two phases (A:B) in the ratio of 80:20 (v/v). First phase (A) consists of a mixture of equal volumes of phosphate buffer (0.01 M in monobasic sodium phosphate and 0.01 M dibasic sodium phosphate) and acetonitrile. The second mobile phase (B) was distilled water. The flow rate was kept at 1 ml/min and the UV absorbance was measured at 318 nm. Various dilutions were made in the mobile phase to prepare a standard curve (Andjelic et al., 2006). The concentration range of linearity was 0.005 mg/ml to 0.1 mg/ml with R^2 value of 0.9999. The method is precise with relative standard deviation of <1.0%. The presence of HPMC or Dowfax did not altered the retention time of indomethacin.

3. Results and discussion

3.1. Model drug and stabilizer selection

Indomethacin was selected due to its low solubility and the potential for polymorphic transitions during processing, as it would be interesting to determine whether microfluidization does in fact cause polymorphic transitions in this drug. Indomethacin has a molecular weight of 357.79 g mol^{-1} and is practically insoluble in water (US Pharmacopoeia, 2006). It has been shown to exist in several polymorphic crystalline forms, out of which α and γ are the most common polymorphs and γ is the most stable form (Legendre and Feutelais, 2004). It has been reported that indomethacin is readily converted into its amorphous form on grinding (Watanbe et al., 2003).

Table 4
Initial mean D90 volume weighted diameter (nm) of nanosuspensions.

Run order	Mean D90 volume weighted diameter (nm)							
	Time (min)							
	15		30		60		90	
	Mean	S.D	Mean	S.D	Mean	S.D	Mean	S.D
1	1053	71	836	39	596	20	494	6
2	1837	96	1389	53	991	10	792	23
3	1379	149	1082	48	854	39	722	30
4	2441	158	1378	119	1008	62	864	62
5	1241	109	944	63	735	35	627	10
6	1884	45	1215	154	974	75	878	14
7	1177	54	830	32	614	4	551	8
8	1141	48	853	49	665	36	561	18
9	1975	159	1497	59	1054	57	944	57
10	973	47	703	43	541	19	473	18
11	1473	57	1144	35	871	30	737	46
12	1333	101	1047	13	766	14	656	14
13	1628	54	1070	16	828	15	665	38
14	1166	33	828	12	645	32	558	18
15	1159	46	908	24	664	39	554	35
16	1211	37	809	45	600	18	547	13
17	2412	181	1599	67	1140	23	897	76
18	1348	80	1040	38	784	16	785	51
19	922	31	692	11	495	15	430	10
20	1848	107	1313	5	953	18	820	19
21	1165	217	894	8	646	32	597	9
22	2023	141	1470	106	1164	20	970	109

S.D: standard deviation.

Table 5
Mean D90 volume weighted diameter (nm) following storage at 4 °C.

Run order	Mean D90 volume weighted diameter (nm)							
	Time (days)							
	Initial		7		14		28	
	Mean	S.D	Mean	S.D	Mean	S.D	Mean	S.D
1	494	6	509	12	535	14	546	8
2	792	23	860	12	886	11	931	23
3	722	30	727	61	753	45	735	11
4	864	62	910	42	938	27	914	22
5	627	10	628	6	632	13	630	23
6	878	14	859	48	921	11	930	28
7	551	8	569	21	563	19	565	17
8	561	18	583	6	574	20	619	22
9	944	57	845	17	890	25	887	53
10	473	18	472	2	480	16	473	13
11	737	46	785	67	738	48	772	17
12	656	14	665	42	662	12	643	21
13	665	38	707	6	712	26	763	19
14	558	18	604	34	580	33	617	23
15	554	35	584	24	612	19	565	7
16	547	13	578	26	574	18	568	13
17	897	76	975	14	1010	37	964	15
18	785	51	742	16	766	13	781	41
19	430	10	449	1	449	2	462	17
20	820	19	868	24	888	44	910	92
21	597	9	601	30	610	20	581	22
22	970	109	991	11	1099	99	996	55

S.D: standard deviation.

Table 6
Mean D90 volume weighted diameter (nm) following storage at 25 °C.

Run order	Mean D90 volume weighted diameter (nm)							
	Time (days)							
	Initial		7		14		28	
	Mean	S.D	Mean	S.D	Mean	S.D	Mean	S.D
1	494	6	503	14	531	29	571	13
2	792	23	902	28	1002	34	1026	43
3	722	30	737	18	766	46	751	10
4	864	62	974	16	1008	52	1027	36
5	627	10	615	8	620	4	618	24
6	878	14	857	112	882	28	886	18
7	551	8	599	33	599	7	592	4
8	561	18	594	18	658	7	721	21
9	944	57	851	27	914	40	910	29
10	473	18	468	11	499	18	495	9
11	737	46	753	45	766	22	759	21
12	656	14	627	8	657	19	648	31
13	665	38	703	19	729	27	761	17
14	558	18	670	16	641	13	674	13
15	554	35	554	19	584	16	553	13
16	547	13	622	28	616	20	656	13
17	897	76	1020	16	1027	71	1029	45
18	785	51	749	10	771	12	746	6
19	430	10	480	10	476	6	489	9
20	820	19	881	58	903	34	962	47
21	597	9	604	14	613	24	563	13
22	970	109	947	26	994	28	947	74

S.D: standard deviation.

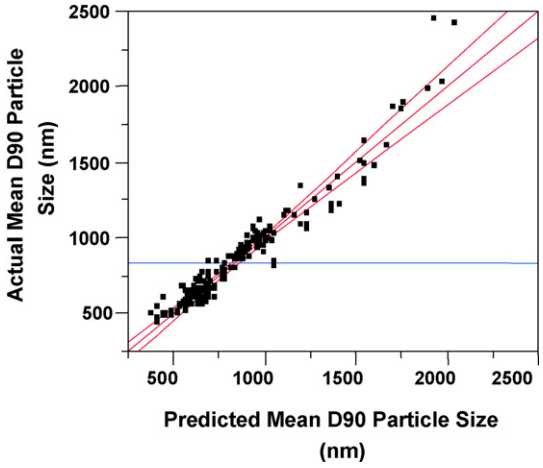

Fig. 1. Actual vs predicted plot for mean D90 particle size.

Selection of potential stabilizers was based on two preconditions: (i) they should belong to different classes, *i.e.* polymeric vs ionic and (ii) they should have minimal effect on drug solubility since we have previously determined (data not shown) that drug solubility in the stabilizer solution plays a significant role in the formation of a stable nanosuspension. Based on these criteria two stabilizers were selected: (i) HPMC E5, a polymer with a molecular weight of approximately 10,000 Da; and (ii) Dowfax 2A1, a small molecule anionic surfactant with a molecular weight of 569 Da.

3.2. Statistical analysis of particle size

Tables 4 to 6 show the results of mean D90 volume weighted particle size along with their standard deviations for all experiments that were part of the experimental design to determine process and formulation design space. Precise particle size distributions were obtained in all experiments as evidenced by the standard devia-

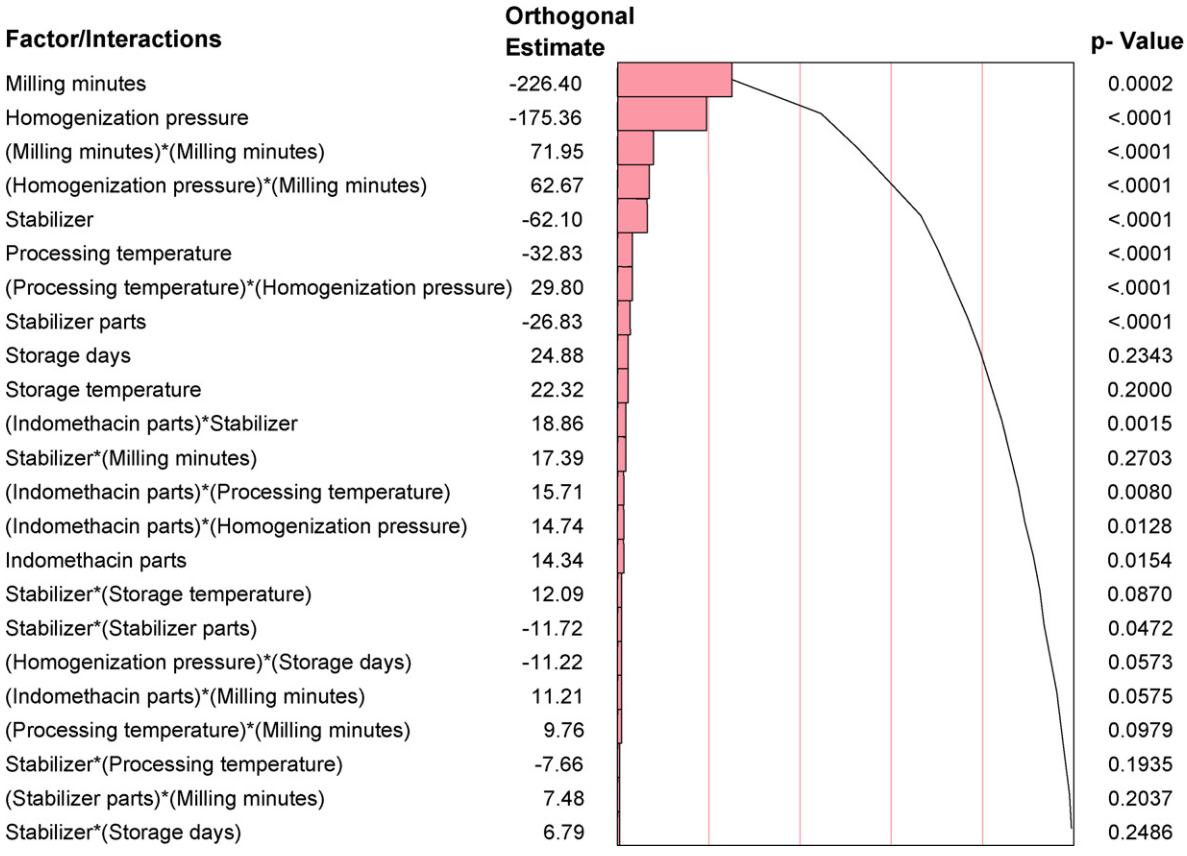

Fig. 2. Pareto plot for mean D90 particle size.

tion values. To deconvolute the individual effects and interactions between the various parameters, the data was statistically analyzed using Minitab and Jump (JMP) software. Multiple linear regression analysis and ANOVA were employed to model the data and develop a mathematical expression. The general form of the mathematical expression is given by the following equation:

$$Y = B_0 + B_1X_1 + B_2X_2 + B_3X_3 + \cdots + B_{12}X_1X_2 + B_{13}X_1X_3 + B_{23}X_2X_3 + \cdots$$

where Y is the dependent variable or response variable such as mean volume weighted particle size; X_1, X_2, X_3,... are the factors or independent variables which affect the process; B_0 is the intercept; B_1, B_2, B_3... and B_{12}, B_{13} and B_{23} are empirically derived coefficients that relate the independent variables X_i or their interactions with the response Y. Fig. 1 shows the relationship between the actual mean D90 volume weighted particle size and the predicted mean D90 volume weighted particle size. A good fit to the data was obtained with a R^2 value of 0.94. Predicted mean D90 volume weighted particle size values were generated using Eq. (1). This analysis is based on the reduced model. Fig. 2 shows the pareto plot for the mean D90 particle size along with the p-values for various factor or interactions. It can be seen that the total time of microfluidization, the homogenization pressure, the type of stabilizer, the concentration of stabilizer, the concentration of indomethacin and the temperature of the product during processing are the critical factors (p-value <0.05) in the formation of nanosuspensions. Two-way interactions between homogenization pressure/milling time, indomethacin concentration/stabilizer, indomethacin concentration/homogenization pressure, indomethacin concentration/processing temperature and homogenization pressure/processing temperature are the significant factors affecting the microfluidization of indomethacin suspensions. In addition, Fig. 2 also shows the rank order of the different variables involved in the formation of indomethacin nanosuspensions along with their interactions with one another. Fig. 3 shows the prediction profiler for a mean D90 volume weighted particle size of approximately 640 nm. It summarizes the major trends of the effects of individual variables on the mean D90 volume weighted particle size of indomethacin as discussed below.

$$\begin{aligned}
&1656.713 + 8.410 \times \text{indomethacin parts} + (-62.097 \times \text{stabilizer}) + (-31.457 \times \text{stabilizer parts}) + \\
&(-3.849 \times \text{processing temperature}) + (-0.051 \times \text{homogenization pressure}) + (-2.355 \times \text{milling minutes}) + \\
&(0.886 \times \text{storage temperature}) + (0.861 \times \text{storage days}) + ((\text{indomethacin parts-5}) \times 11.056 \times \text{stabilizer}) + \\
&(0.921 \times (\text{indomethacin parts-5}) \times (\text{processing temperature-15})) + (0.002 \times (\text{indomethacin parts-5}) \times \\
&(\text{homogenization pressure-14,000})) + (0.242 \times (\text{indomethacin parts-5}) \times (\text{milling minutes-73.5})) + \\
&(-13.740 \times (\text{stabilizer parts-2}) \times \text{stabilizer}) + (-0.898 \times (\text{processing temperature-15}) \times \text{stabilizer}) + \\
&(0.297 \times (\text{milling minutes-73.5}) \times \text{stabilizer}) + (1.177 \times (\text{storage temperature-10.3}) \times \text{stabilizer}) + \\
&(0.811 \times (\text{storage days-9.8}) \times \text{stabilizer}) + (0.322 \times (\text{stabilizer parts-2}) \times (\text{milling minutes-73.5})) + (0.001 \times \\
&(\text{processing temperature-15}) \times (\text{homogenization pressure-14,000})) + (0.042 \times (\text{processing temperature-15}) \times \\
&(\text{milling minutes-73.5})) + (0.001 \times (\text{homogenization pressure-14,000}) \times (\text{milling minutes-73.5})) + (-0.0003 \times \\
&(\text{homogenization pressure-14,000}) \times (\text{storage days-9.8})) + (0.183 \times \text{milling minutes} \times (\text{milling minutes-73.5}))
\end{aligned} \quad (1)$$

where stabilizer equals 1 for Dowfax 2A1 and − 1 for HPMC E5.

3.2.1. Effect of process and formulation variables

3.2.1.1. Concentration of indomethacin. Higher concentrations of indomethacin in the suspension produce suspensions with higher mean D90 particle size. This factor (indomethacin concentration) also interacts with other factors namely homogenization pressure, stabilizer type and processing temperature (Fig. 2). The interaction of indomethacin concentration with stabilizer (Dowfax 2A1) is the most significant of these interactions and has a p-value of 0.0015. On examination of various two-way interactions (Fig. 4), it is apparent that a lower particle size is obtained at lower concentrations of indomethacin when Dowfax 2A1 was used as a stabilizer. On the other hand, no difference in particle size was observed when HPMC E5 was used as a stabilizer. It may be attributed to the higher amounts of Dowfax 2A1 being required, due to its small molecular size, for covering the surface of the nanoparticles as compared to HPMC. Lower particle size was observed at lower indomethacin concentrations when higher processing temperatures or homogenization pressures were used in the process. The power input is identical for both the cases (lower and higher concentration of indomethacin), therefore better particle size reduction was achieved with smaller number of particles (lower concentration) than with large number of particles. This is also supported by the observation that larger mean D90 particle size was observed after 90 min of milling with formulations made with higher drug concentration.

Table 7
Solubility of indomethacin in various solutions at 25 °C.

Media	Solubility (μg/ml) (mean ± S.D)
Distilled water	0.94[a]
0.5% (w/v) HPMC E5	15.21 ± 0.70
0.5% (w/v) Dowfax 2A1	18.27 ± 0.02

[a] Wishart et al. (2006).

3.2.1.2. Stabilizer type. The type of stabilizer was determined to have a significant effect on nanosuspension particle size ($p < 0.001$). Both Dowfax 2A1 and HPMC E5 were able to form nanosuspensions. A smaller particle size was obtained when Dowfax 2A1 was used as compared to HPMC E5. Besides specific interaction with the drug, stabilizer efficacy also depends on the effect of the stabilizer on drug solubility, viscosity of the stabilizer solution and diffusion of the stabilizer molecules to the interface (Sepassi et al., 2007). Table 7 shows the solubility of indomethacin in a 0.5% (w/v) aqueous solution of Dowfax 2A1 and HPMC E5. Both stabilizers show a similar increase in the solubility of indomethacin. HPMC E5 is a low viscosity polymer and moreover the concentrations of HPMC E5 used in the preparation of the nanosuspensions were very low. Therefore, the viscosities of the HPMC E5 suspensions were comparable to those of Dowfax 2A1 suspensions. Thus, it can be speculated that the slower diffusion of the bulky HPMC E5 molecules (molecular wt. 10,000 Da), as compared to Dowfax 2A1 molecules (molecular wt. 569 Da), to the newly formed interfaces may be responsible for the higher particle size observed in HPMC E5 based formulations.

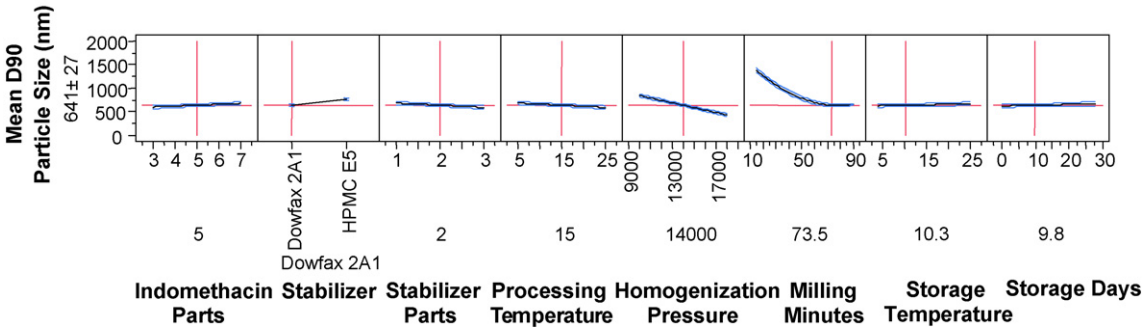

Fig. 3. Prediction profiler for mean D90 particle size.

3.2.1.3. Stabilizer concentration. Decrease in stabilizer concentration increased the mean D90 particle size of indomethacin nanoparticles. A minimum concentration of stabilizer is necessary to form a stable nanosuspension (Rasenack and Muller, 2002). Lack of stabilization due to incomplete coverage of the rapidly generating newer nanoparticles at lower stabilizer concentration may be responsible for higher particle size at lower stabilizer concentration. Slower migration of the stabilizer molecules at lower concentrations also leads to inadequate stabilization of the newly formed interfaces resulting in higher particle size. No significant interactions were observed between the stabilizer concentration and the other process and formulation variables.

3.2.1.4. Processing temperature. Nanosuspension particle size showed an inverse dependence on the processing temperature. At 25 °C smaller particle sizes were achieved as compared to 4 °C. This can be explained as follows: the enormous energy applied during processing causes the solid state, suspended particles of indomethacin to go into solution and form a supersaturated solution. Within the interaction chambers of the microfluidizer a dynamic equilibrium exists between the supersaturated solution of indomethacin and the suspended particles which govern the particle size. At low temperatures indomethacin exhibits lower solubility as compared to higher temperatures. As a result, lower saturation solubility is expected at lower processing temperatures. Lower saturation solubility results in re-crystallization of the excess dissolved drug onto the existing particles resulting in a higher particle size. At higher temperatures the system is able to maintain a higher saturation solubility which leads to a smaller size. This explains the interaction observed between temperature and homogenization pressure.

As shown in Fig. 4 the effect of temperature is more pronounced at lower pressures than at higher pressures. At 10,000 psi a lower particle size is obtained at 25 °C compared to 4 °C. At 10,000 psi the rate of increase of temperature within the interaction chamber was very slow and the temperature inside the interaction chamber (which cannot be controlled accurately due to instrument limitations) can be assumed to be approximately equal to that of the bulk suspension (controlled accurately within ±1 °C). However, at higher pressures, though the temperature of the bulk suspension was maintained within ±1 °C of the desired value, the rate of increase of temperature within the interaction chamber was not controlled by the bulk temperature. The outside of the interaction chamber became warm at both temperatures, indicating that the temperature inside the interaction chamber was independent of the bulk temperature. At high pressures of 18,000 psi, the temperature inside the interaction chamber was a function of pressure only and can be assumed to be equal in both cases. This explains why similar particle sizes were obtained at two different processing temperatures when high pressure was used for nanosuspension preparation.

3.2.1.5. Milling time. The milling time is the most important factor, governing the process of microfluidization, identified in this study (Fig. 2). However, particle size reduction by microfluidization does not depend linearly on milling time as shown by the significant squared estimate term in Fig. 2. The milling process is also dependent on the pressure employed (Fig. 4). At low pressure the process is more gradual as compared to that at high pressure. At low pressure particle size decreases linearly up to approximately 50 min and then the rate decreases until about 70 min after which no change in the particle size is obtained and a plateau is reached. At high pressure the slope of particle size reduction is much steeper initially when compared to that at low pressures. After about 30 min into the milling process, the rate of particle size reduction decreases and finally plateaus after 60 min. However, after approximately 75–85 min a positive slope is observed indicating an increase in particle size which is usually more prominent at high pressures. After extended periods of milling, particle size growth can occur due to various reasons such as shortage of excess stabilizer, agglomeration due to increased surface energy and re-crystallization of the dissolved drug (Illig et al., 1996; Mackin et al., 2002a).

3.2.1.6. Homogenization pressure. Homogenization pressure is the second key variable that has prominent effect on particle size reduction of indomethacin (Fig. 2). The higher the pressure, the smaller is the particle size (Fig. 3). The interactions between homogenization pressure and the other variables are explained in each of the above sections.

3.2.2. Contour plots

Contour plots are a graphical representation of the relationship between three variables in two dimensions. These can be very helpful in illustrating the complete picture of the effect of two independent variables simultaneously (interactions) on the third variable (dependent variable). Fig. 5 shows the actual and predicted contour plots in relation to milling minutes, homogenization pressure and mean D90 volume weighted particle size for Dowfax 2A1 and HPMC E5. It can be seen that the predicted plots are very similar to the plots derived from the actual data. Contour plots provide valuable information about the design space of a process and facilitate in establishment of process specifications. For example, for HPMC E5 stabilizer a mean D90 particle size of less than 1000 nm can be achieved by milling for just 40 min at 18,000 psi or 80 min at 12,000 psi. Thus depending on other factors, such as the physical and chemical stability of the drug and stabilizer, the process can be optimized to achieve the desired product. Similar contour plots can be generated between other variables studied in the design of experiments. A thorough understanding of the process and formulation variables, which is the foremost goal of the quality by design initiative, has been shown to greatly assist in initial regulatory approvals and post-approval changes (Yu, 2008).

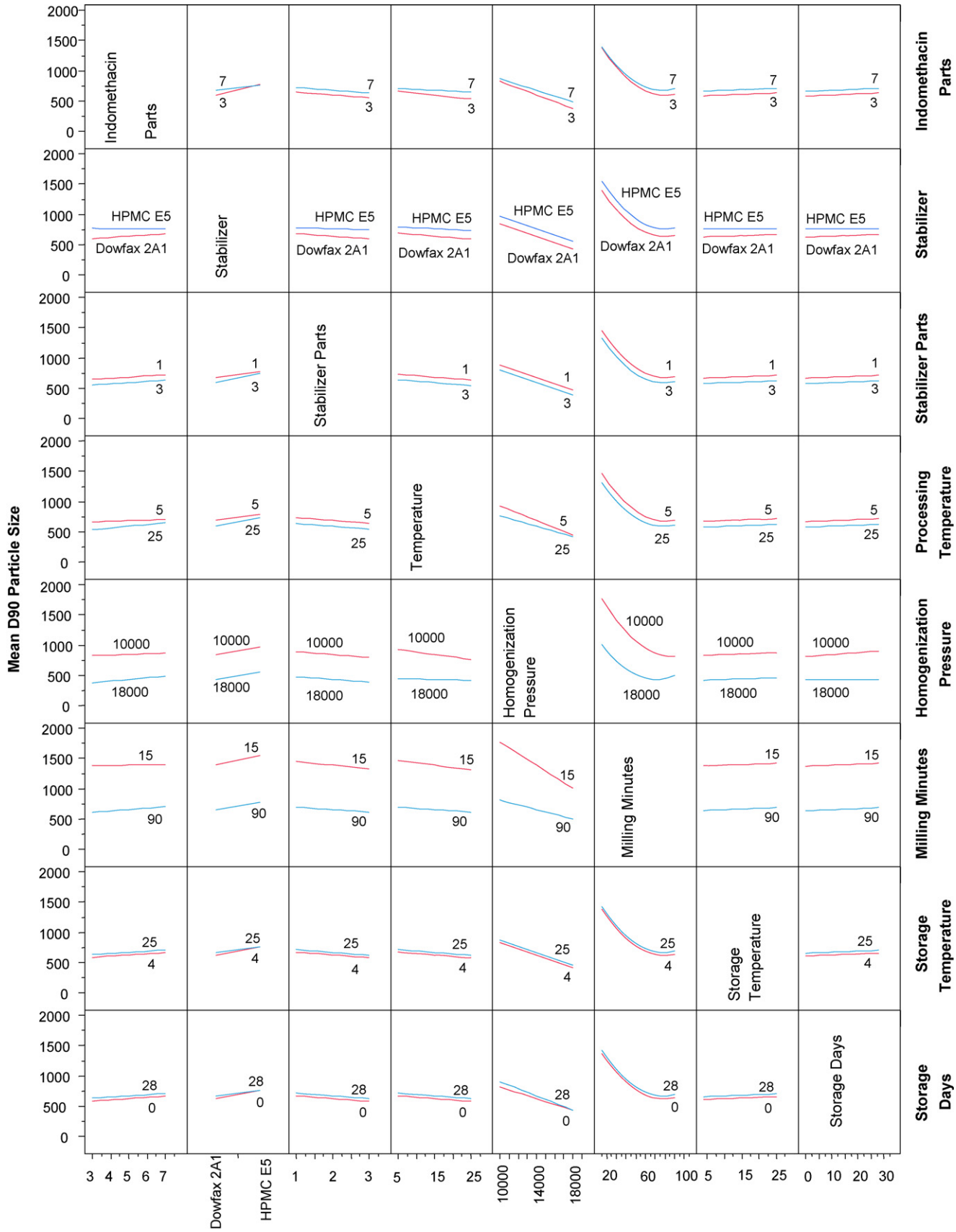

Fig. 4. Two-way interactions for mean D90 particle size.

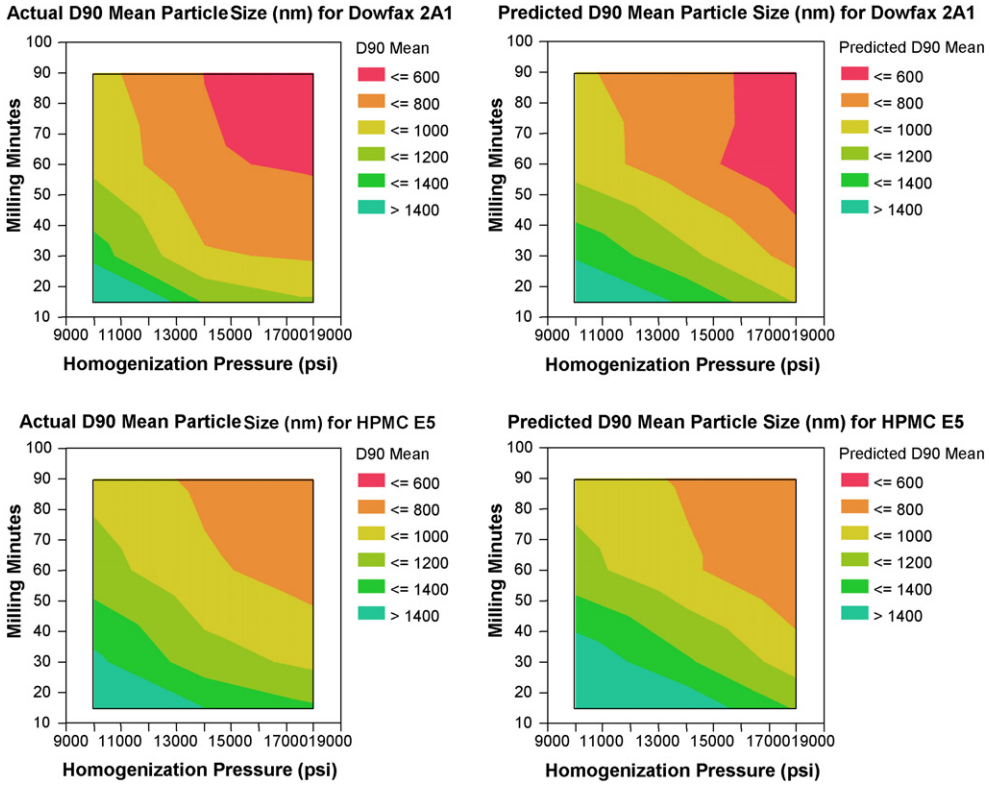

Fig. 5. Contour plots of HPMC E5 and Dowfax 2A1.

3.2.3. *Physical stability*

Particle size reduction techniques (Ticehurst et al., 2000) are extremely inefficient unit operations (Parrot, 1990) that involve high energy input. These processes are often associated with the generation of amorphous regions or crystal defects (Saleki-Gerhardt et al., 1994; Ward and Schultz, 1995; Mackin et al., 2002b; Begat et al., 2003) on the surface of the micro/nanosized material resulting in a highly activated surface (Hütterauch et al., 1985). Such galvanized regions are extremely difficult to detect with standard techniques such as differential scanning calorimetry and X-ray powder diffraction (Ticehurst et al., 2000). However, restructuring of the surface due to re-crystallization of amorphous regions or

Table 8
Mean zeta potential of nanosuspensions following storage at 4 °C.

Run order	Mean zeta potential (mV)							
	Time (days)							
	Initial		7		14		28	
	Mean	S.D	Mean	S.D	Mean	S.D	Mean	S.D
1	−70.32	4.22	−65.36	2.08	−63.08	1.28	−75.86	3.58
2	−68.58	8.24	−73.20	3.61	−78.03	7.40	−74.81	1.69
3	−13.30	2.44	−8.18	2.81	−7.87	1.54	−9.98	4.78
4	−55.95	5.08	−57.81	5.47	−65.78	1.15	−52.37	1.59
5	−5.80	2.40	−11.48	0.27	−7.26	1.61	−19.41	4.16
6	−8.30	1.40	−9.21	9.86	−12.72	4.08	−9.93	3.01
7	−65.47	9.39	−68.59	5.87	−66.84	7.19	−61.37	2.74
8	−52.83	3.92	−55.05	3.25	−47.91	3.83	−61.85	2.11
9	−11.77	1.16	−12.79	0.80	−10.09	3.38	−14.80	1.59
10	−57.93	7.28	−55.73	1.35	−57.35	6.76	−55.71	5.22
11	−7.08	1.83	−13.36	4.87	−6.05	3.28	−5.81	0.43
12	−16.84	4.00	−8.63	1.68	−4.19	11.09	−7.90	3.43
13	−77.00	4.16	−76.17	3.51	−72.78	11.44	−66.20	4.78
14	−62.01	4.78	−58.24	5.32	−56.89	6.00	−65.59	9.03
15	−8.71	3.36	−5.86	0.89	−10.15	3.06	−8.01	2.13
16	−67.43	3.98	−67.94	6.54	−62.06	1.66	−63.87	4.64
17	−5.72	4.97	−7.59	2.70	−10.43	4.79	−6.88	3.08
18	−11.10	5.35	−11.97	0.51	−13.48	2.30	−13.24	3.87
19	−70.56	2.45	−73.79	1.59	−72.45	7.31	−65.99	9.14
20	−64.49	7.19	−64.81	9.59	−58.51	8.03	−60.73	5.63
21	−5.76	3.21	−7.95	0.66	−11.23	3.03	−16.05	7.15
22	−12.45	1.26	−9.32	10.12	−12.59	1.25	−10.08	4.83

S.D: standard deviation.

Table 9
Mean zeta potential of nanosuspensions following storage at 25 °C.

Run order	Mean zeta potential (mV)							
	Time (days)							
	Initial		7		14		28	
	Mean	S.D	Mean	S.D	Mean	S.D	Mean	S.D
1	−70.32	4.22	−74.80	3.09	−72.29	5.65	−81.63	10.67
2	−68.58	8.24	−79.00	2.63	−77.44	3.29	−73.88	9.44
3	−13.30	2.44	−12.65	6.11	−12.80	3.13	−11.56	4.73
4	−55.95	5.08	−60.37	4.43	−63.25	5.33	−56.90	4.26
5	−5.80	2.40	−13.80	6.09	−12.47	2.38	−15.86	0.68
6	−8.30	1.40	−5.48	1.35	−8.73	1.21	−11.58	5.87
7	−65.47	9.39	−66.87	3.37	−68.17	8.44	−64.83	1.77
8	−52.83	3.92	−65.71	3.16	−59.56	2.72	−61.37	3.47
9	−11.77	1.16	−13.16	3.65	−8.40	1.39	−14.43	4.00
10	−57.93	7.28	−60.99	4.93	−62.80	4.28	−51.21	9.41
11	−7.08	1.83	−7.64	2.03	−10.86	7.13	−7.10	4.83
12	−16.84	4.00	−6.00	2.05	−11.56	1.69	−8.04	3.03
13	−77.00	4.16	−74.33	7.52	−68.72	8.06	−67.47	2.43
14	−62.01	4.78	−60.29	4.62	−64.12	4.43	−59.74	7.74
15	−8.71	3.36	−8.29	0.86	−12.55	4.21	−9.02	2.77
16	−67.43	3.98	−67.89	1.84	−67.03	3.25	−65.49	4.18
17	−5.72	4.97	−7.28	3.10	−10.11	1.04	−6.16	1.93
18	−11.10	5.35	−8.46	2.15	−14.38	5.00	−14.28	5.40
19	−70.56	2.45	−69.31	4.59	−71.86	6.81	−70.27	0.34
20	−64.49	7.19	−75.33	7/27	−63.67	2.90	−66.12	6.44
21	−5.76	3.21	−7.42	3.95	−9.37	8.41	−14.62	0.94
22	−12.45	1.26	−7.83	9.10	−8.03	2.40	−12.69	1.02

S.D: standard deviation.

re-ordering of the lattice is often manifested as physical or chemical instability (Otsuka and Kaneniwa, 1990; Ticehurst et al., 2000; Shalaev et al., 2002; Brodka-Pfeiffer et al., 2003; Ohta and Buckton, 2005) in formulations on storage. To study this aspect with respect to microfluidization processing, all the batches in the design of experiments were kept at 4 °C and 25 °C for 28 days.

Tables 5 and 6 show the mean D90 volume weighted particle size data of all batches on storage. In order to evaluate the potential effect of processing and formulation variables on the nanosuspension stability, this data was also included in the final analysis. Most of the processing or formulation variables did not exhibit any significant effect on the physical stability of indomethacin nanosuspensions (Fig. 4). Formulations made with Dowfax 2A1 were more susceptible to increase in particle size on storage at higher temperature than HPMC based formulations (Fig. 4). This is due to the polymeric nature of HPMC, which makes the adsorption relatively irreversible compared to the dynamic adsorption/desorption of the small molecule (Dowfax 2A1) at high temperatures (Walstra, 1983).

Only a weak interaction is observed between homogenization pressure and storage days as indicated by a *p*-value of 0.0573 (Fig. 2). An interesting feature, apparent from Fig. 4, is that the mean particle size of the batches prepared at 10,000 psi increased on storage, whereas the mean particle size of batches prepared at 18,000 psi remained unchanged. Ticehurst et al. (2000) observed an increase in the particle size of revatropate hydrobromide, post-micronization, on storage. This was attributed to agglomeration of the particles as a result of re-crystallization at amorphous/disordered regions generated during the grinding process.

In the present case, it can be speculated that at low homogenization pressure the energy imparted to the system was not enough to cause complete particle size reduction. Instead activated regions were generated which may be responsible for agglomeration and hence particle size increase on storage. At high homogenization pressures the energy imparted to the system was sufficient to break down the particles completely. Accordingly, there was a lower tendency for the generation of activated regions and therefore these suspensions were stable with respect to particle size.

3.3. Statistical analysis of zeta potential

Tables 8 and 9 report the mean zeta potential before and after storage at 4 °C and 25 °C for 28 days for all batches prepared according to the design of experiments. The initial mean zeta potential varied from −5.72 mV to a maximum of −77.00 mV (−5.72 mV to −16.84 mV for HPMC E5 batches −52.83 mV and −77.00 mV for Dowfax 2A1 batches). ANOVA and multiple linear regression analysis were used to analyze the mean zeta potential data. A correlation with R^2 value of 0.99 was obtained between the actual mean zeta potential vs the predicted mean zeta potential for the full model analysis (Fig. 6). Fig. 7 shows the pareto plot of mean zeta potential along with the *p*-values for different parameters affecting the mean zeta potential. Stabilizer type and concentration are the most significant factors governing the mean zeta potential values (*p*-values less than 0.0001). Fig. 8 illustrates the main effect of the different variables on the mean zeta potential of the indomethacin nanosuspensions.

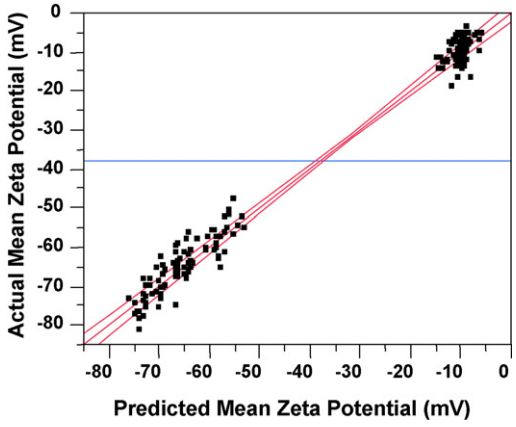

Fig. 6. Actual vs predicted for mean zeta potential.

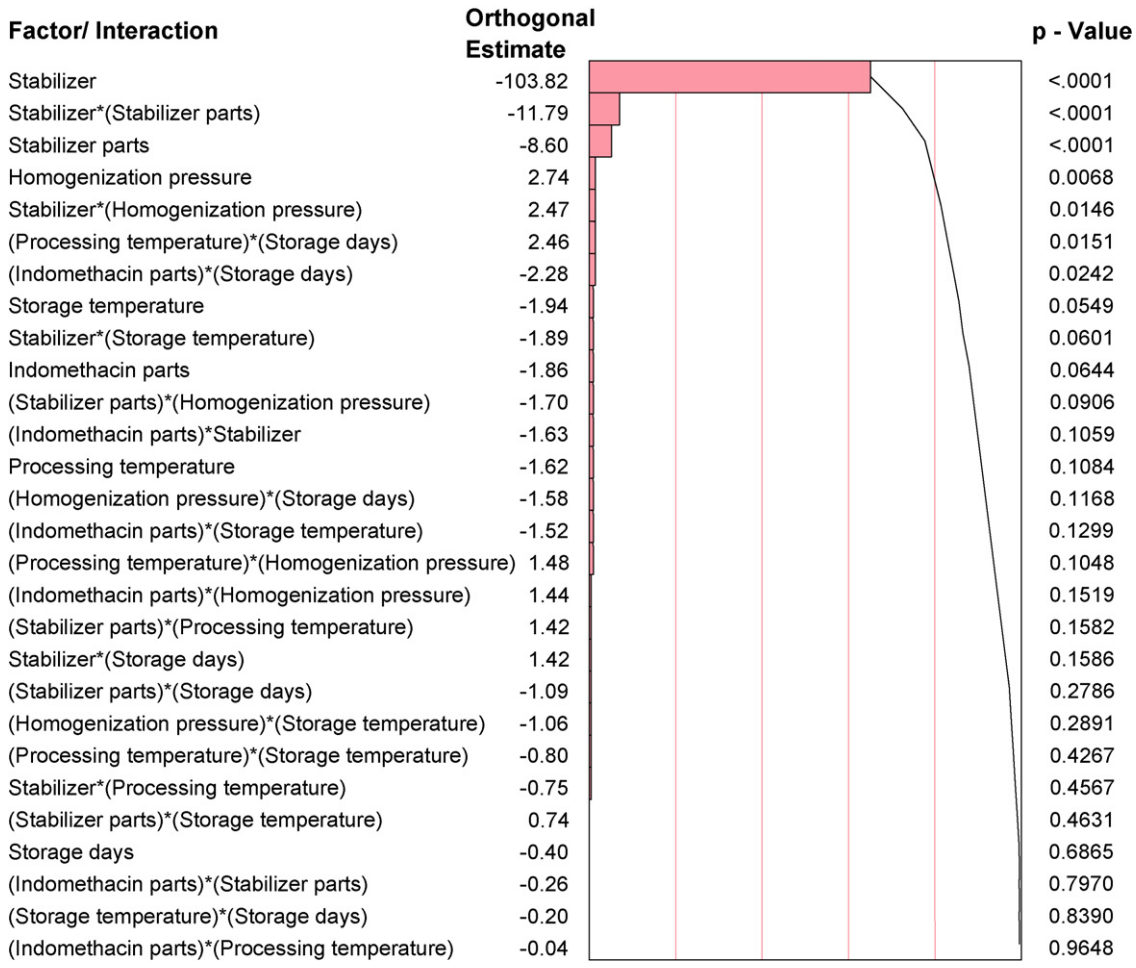

Fig. 7. Pareto plot for mean zeta potential.

Batches made with Dowfax 2A1 had more negative mean zeta potential values as compared to HPMC E5 batches. This is a result of the anioinic nature of Dowfax 2A1 which imparts a negative charge to indomethacin particles upon adsorption. Indomethacin is a carboxylic acid derivative and ionization of the acid group in an aqueous environment results in a net negative charge. Whereas, adsorption of HPMC E5 molecules (non-ionic surfactant) decreases the net charge due to charge shielding effects and reduced charge at the plane of shear. The homogenization pressure also appears to play a role (p-value <0.05) in determining the zeta potential, although its overall influence on the zeta potential is masked by the type and concentration of stabilizer (Fig. 7). Similarly in the full model analysis interactions between homogenization pressure/stabilizer, storage days/temperature of processing and storage days/concentration of indomethacin appears to be significant (Fig. 7).

Fig. 9 summarizes the effect of two-way interactions on the mean zeta potential across all parameters. The majority of the graphs are parallel, except that between stabilizer (Dowfax 2A1) and homogenization pressure, suggesting a general lack of interaction between the different variables with respect to their influence on the zeta potential. At homogenization pressures of 10,000 psi Dowfax formulations exhibited more negative zeta potential values than formulations made at 18,000 psi. An explanation for this is that

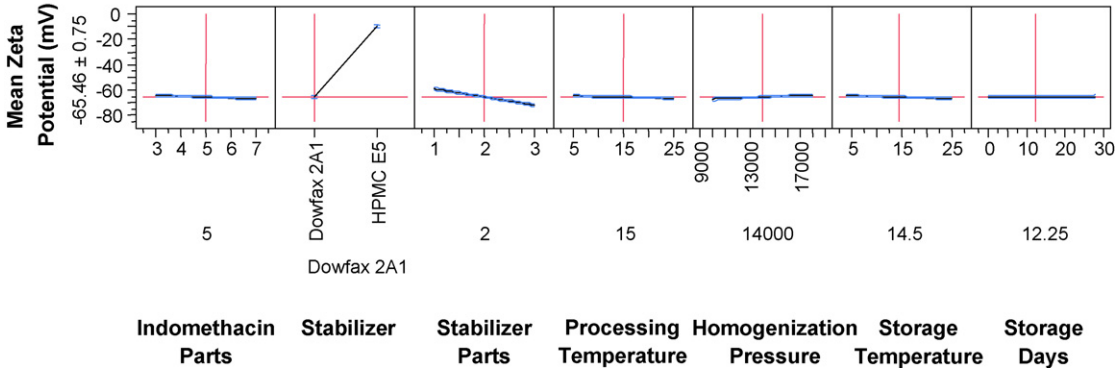

Fig. 8. Prediction profiler for mean zeta potential.

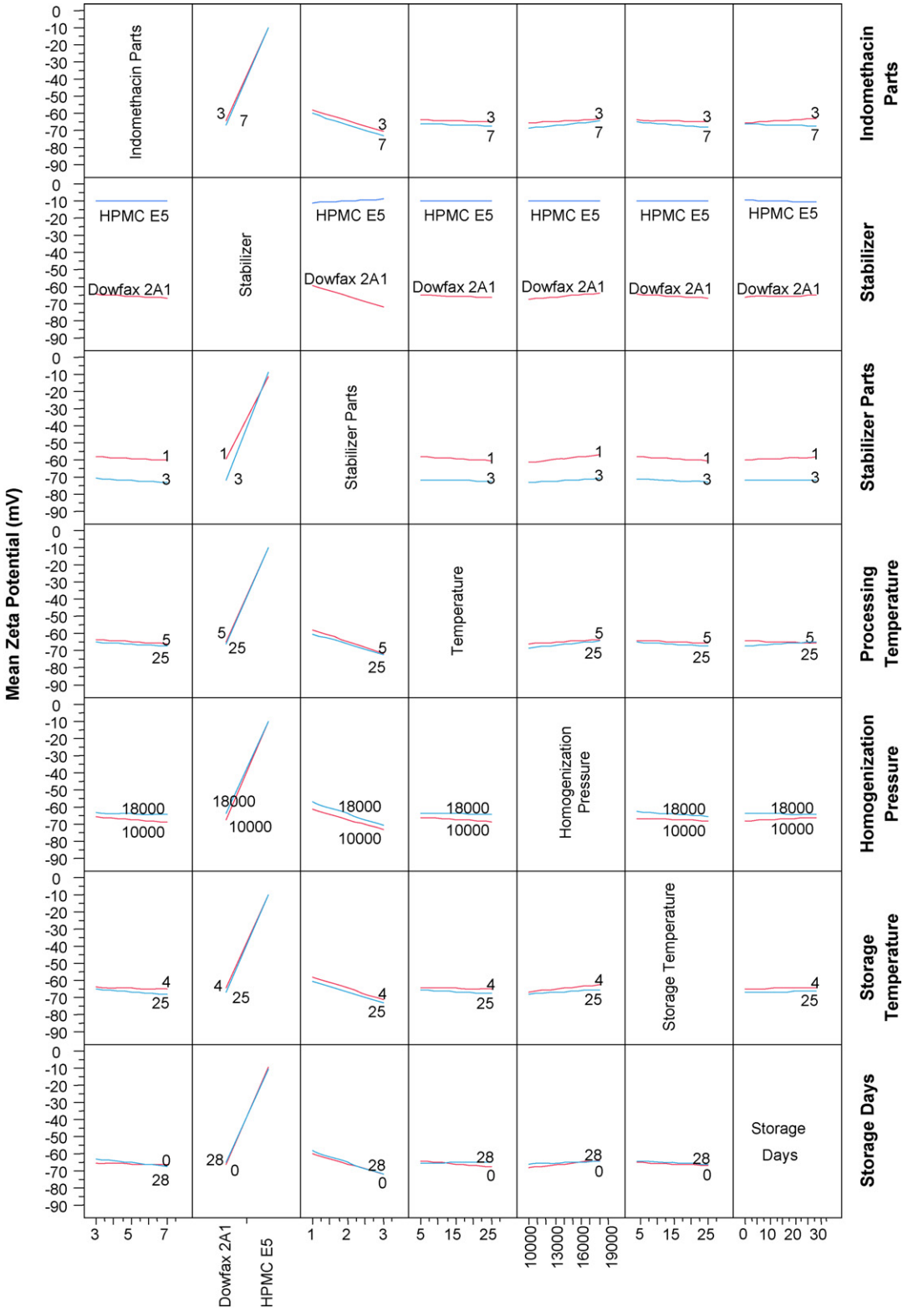

Fig. 9. Two-way interactions for mean zeta potential.

at low pressure (10,000) the particle size of the nanosuspensions was large compared to those made at higher pressure (18,000 psi). Consequently, the total surface area available for adsorption in nanosuspensions made at lower pressures was smaller compared to those prepared at higher pressures. Therefore, at a given concentration, assuming that the surface is not fully packed with the surfactant molecules, more surfactant molecules will be adsorbed per unit area in the case of larger particles as compared to smaller particles. Higher adsorption density should impart greater negative charge and hence larger negative zeta potential values will be observed in the case of nanosuspensions with larger particles (suspensions made at 10,000 psi). This assumption is supported by the observation that increase in Dowfax 2A1 concentration decreases the zeta potential (becomes more negative) (Fig. 9) indicating that

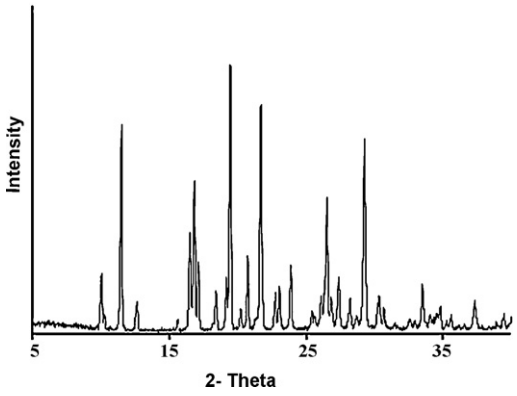

Fig. 10. X-ray diffraction patterns of indomethacin (γ polymorph).

the surfactant layer is not densely packed in the concentration range studied. No effect of homogenization pressure and stabilizer concentration was seen on formulations made with HPMC E5 as the stabilizer (Fig. 9). The zeta potential of the batches did not show any significant changes on storage for 28 days at 4 °C and 25 °C.

3.4. Powder X-ray diffraction

X-ray powder diffraction of all the 22 nanosuspension batches prepared according to the design of experiments was performed. Diffraction peaks of all the batches were in agreement with that of the crystalline γ polymorph, which was the starting material (Fig. 10). Figs. 11 and 12 show the representative X-ray powder diffraction of an HPMC E5 and Dowfax 2A1 batch immediately after preparation (labeled initial). No new peaks or halo could be detected in the X-ray profiles confirming the absence of any polymorphic changes (conversion to α form or generation of amorphous form) during the processing. Although conversion of the stable γ polymorph to the less stable α form and amorphous form have been reported under high pressure (Okumura et al., 2006) and after milling (Otsuka et al., 1986), all these methods involve milling of the indomethacin in a dry state. However, humidity (Watanbe et al., 2001) and energy input (Desprez and Descamps, 2006) have been shown to reverse these polymorphic changes which result in reversion of the less stable forms (α form and amorphous form) to the stable γ form. Figs. 11 and 12 show representative X-ray profiles following storage for 28 days at 4 °C and 25 °C for HPMC E5 and Dowfax 2A1 batch, respectively. No changes in the X-ray patterns were observed confirming that the formulations were stable for approximately one month.

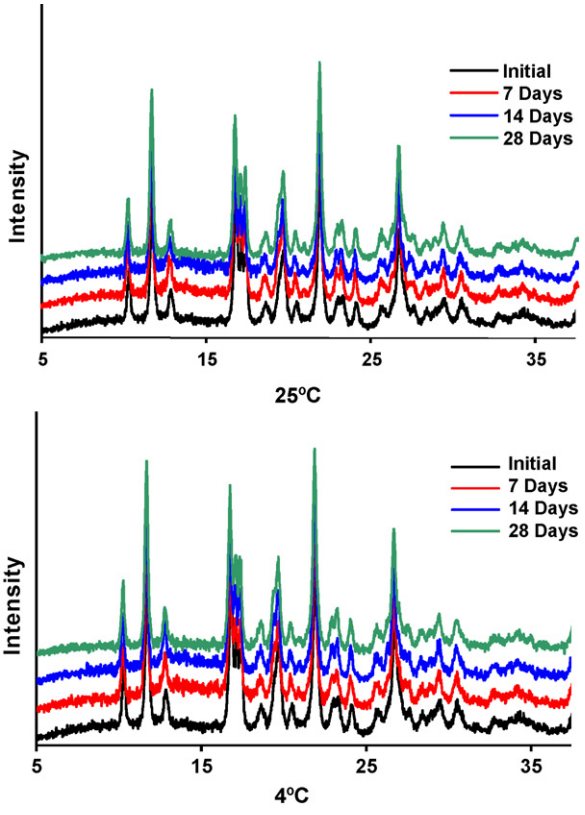

Fig. 11. Representative X-ray diffraction patterns of HPMC E5 nanosuspensions following storage at 25 °C and 4 °C (bottom to top: initial, 7 days, 14 days, 28 days).

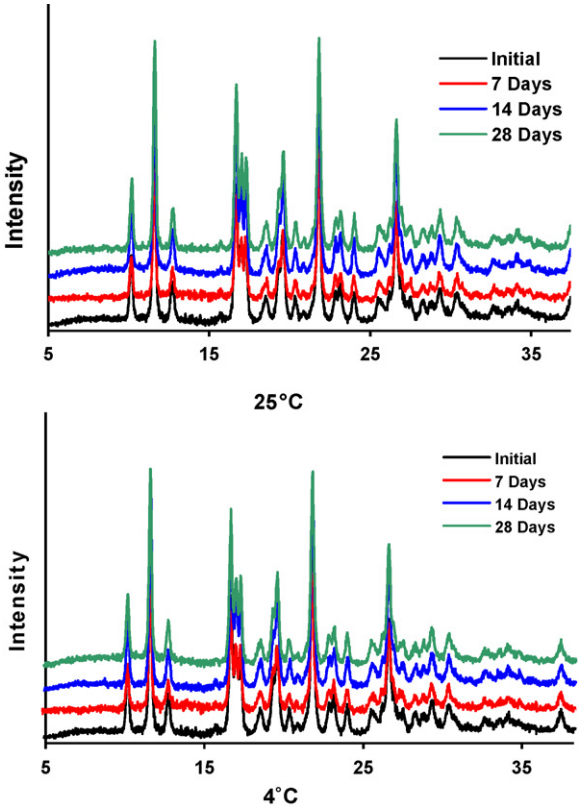

Fig. 12. Representative X-ray diffraction patterns of Dowfax 2A1 nanosuspensions following storage at 25 °C and 4 °C (bottom to top: initial, 7 days, 14 days, 28 days).

4. Conclusions

This study demonstrated the usefulness of the quality by design approach, encompassing the amalgamation of such scientific techniques as DOE, multi factor data analysis and ANOVA, to gain a comprehensive understanding of the preparation and processing of nanosuspensions via microfluidization. Milling time, microfluidization pressure, stabilizer type, processing temperature and stabilizer concentration were identified as critical parameters affecting the formation of indomethacin nanoparticles. Moreover, this quality by design approach facilitated the elucidation of various two-way interactions between independent variables which are impossible to detect with the conventional one factor at a time methodology. Both ionic as well as steric stabilization were effective in stabilizing the nanosuspensions. No correlation was found between zeta potential and nanosuspension stability. Therefore, it can be concluded that as long as adequate protection is provided by either type of stabilizer, no minimum zeta potential is necessary

for achieving stable nanosuspensions. No change in the physical form of the drug was observed on storage for four weeks indicating that the suspensions were stable for the time period studied.

Acknowledgements

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