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Journal of Chromatography A, 917 (2001) 63–73

JOURNAL OF CHROMATOGRAPHY A

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Investigation of pharmaceutical high-performance liquid chromatography assay bias using experimental design

Jian-Ge Chen*, Kelly Glancy, Xiaohong Chen, Mark Alasandro

DuPont Pharmaceuticals Company, Wilmington, DE 19880-0353, USA

Received 20 December 2000; received in revised form 13 February 2001; accepted 19 February 2001

Abstract

This article presents a systematic approach to investigate, document, and eliminate pharmaceutical HPLC assay bias using experimental design. This is the first article to describe the application of experimental design in the area of assay bias. It is found that both formulation and analytical variables can contribute to pharmaceutical HPLC assay bias using model compounds and formulations.

Keywords: Assay bias; Experimental design; DMP 543; XP 280

1. Introduction

Experimental design is a powerful and valuable tool for pharmaceutical dosage development, and it has been widely used in pharmaceutical research. It has been used successfully in formulation and process development of dosage forms [1–4] and in analytical method development and validation.

A literature search shows many experimental design applications in analytical method development and validation, especially in the area of separation sciences. Experimental design has been used for separation optimization in reversed-phase [5–7], ion-pair reversed-phase [8,9], micellar [10], chiral [11], and normal-phase [12] high-performance liquid chromatography (HPLC), and in gas [13–15], ion [16–19], supercritical fluid [20,21], size-exclusion [22], displacement [23], extraction [24], and micellar electrokinetic [25] chromatographies. Other applications in separation sciences include recovery study of analyte in urine and plasma samples [26], derivatization reaction procedures [27–29], and detection limit optimization in gas chromatography [30,31], ruggedness tests in reversed-phased HPLC [32–34], and liquid chromatography–mass spectrometry (LC–MS) interface optimization [35]. However, there is no literature on the application of experimental design in the investigation of pharmaceutical HPLC assay bias.

For pharmaceutical products, an acceptable HPLC assay accuracy is typically ±3% of target concentration. This is not stated in regulatory guidelines; however, it is a good scientific and regulatory practice to investigate systematic errors and to eliminate assay bias. On the other hand, practical implications of assay bias depend highly on the

*Corresponding author. Present address: Schering-Plough Research Institute, Kenilworth, NJ 07033, USA.

E-mail address: jian-ge.chen@spcorp.com (J.-G. Chen).

0021-9673/01/$ – see front matter
PII: S0021-9673(01)00670-7

content uniformity of the product and needs to be evaluated on a case-by-case basis. For example, a 0.1% assay bias would not have any relevance on a product with a 5% content uniformity.

Several factors can contribute to pharmaceutical HPLC assay bias. These variables include hydrophobicity of the active drug substance, the amount and type of each excipient in the formulation, size of the capsule shells or tablet coating materials, amount of organic solvent and inorganic buffer in the sample solvent, and amount of solvent used in preparing the sample. These variables can introduce assay bias by changing the physical properties of the solvent such as density and viscosity and by selective uptake or adsorption of aqueous or organic components of the sample solvent and/or the active drug substance. Change in physical properties of the sample solvent affects the amount of sample injected and/or the chromatographic peak shapes of the analytes, and may therefore, lead to assay bias. Selective uptake and adsorption of solvent components and drug substance by insoluble excipient components can decrease or increase the drug concentration in the sample solution and hence, lead to assay bias. The effects of these variables are more pronounced in assay methods for very potent products because their assays require very small amounts of sample solvent, hence larger excipient to solvent ratio, to have sufficient sensitivity and precision.

Whether or not it is possible to detect an assay bias depends on the standard deviation of the test method and the number of replicates used to determine that standard deviation. If the 95% confidence interval [mean$\pm t(\sigma/\sqrt{n})$] covers 100% recovery, the method is said to have no bias. For example, if $n=9$ and the range (mean$\pm 0.77\sigma$) covers 100% recovery, the method is said to have no assay bias.

It is unrealistic to determine assay bias by trial and error when there are three or more variables, especially in the presence of quadratic and interaction effects. In this article, a systematic approach to investigate and document assay bias is demonstrated using experimental design with the ECHIP software, and using a hydrophobic (DMP 543) and a hydrophilic (XP 280) developmental drug in a model formulation.

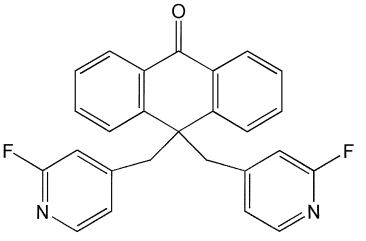
2. Experimental

2.1. Materials

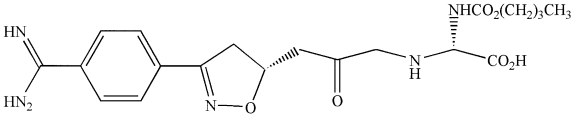
DMP 543 and XP 280 drug substances were manufactured by Chemical Process R&D of DuPont Pharmaceuticals (Wilmington, DE, USA). All solvents were HPLC grade. Acetonitrile was purchased from EM Science (Gibbstown, NJ, USA). Purified water was obtained from Milli-Q Plus ultra-pure water system (Millipore, Milford, MA, USA). Monobasic sodium phosphate monohydrate was purchased from Fisher Scientific. Lactose monohydrate was purchased from Foremost (Rothschild, WI, USA). Magnesium stearate was purchased from Mallinckrodt (St. Louis, MO, USA). Explotab was purchased from Penwest Pharm (Lestrem, France).

2.2. Model compounds and formulation

Structures of model compounds (DMP 543 and XP 280) are given in Fig. 1. DMP 543 is a hydrophobic drug with an anticipated therapeutic dose of 25 to 100 μg. XP 280 is a hydrophilic drug with an anticipated therapeutic dose of 100 to 400 μg. The model capsule formulation used in this



DMP 543



XP 280

Fig. 1. Structures of model compounds used in this study.

study contains lactose monohydrate as the diluent, magnesium stearate as the lubricant, and Explotab as the disintegrant. The filled mass for each capsule is 120 mg. The mass of the active pharmaceutical ingredient as compared to the total filled mass is negligible (i.e., 0.10 mg XP 280 in 120 mg and 0.025 mg DMP 543 in 120 mg excipient).

2.3. Sample preparation

DMP 543 stock spiking solution was prepared by accurately weighing 50 mg of DMP 543 reference standard into a 250-ml volumetric flask, add about 100 ml of solvent (i.e., 50%, v/v, acetonitrile in water), shaking to dissolve, and bring to volume with solvent. The spiking solution was prepared by pipetting 10 ml of the stock solution into a 250-ml volumetric flask and bring to volume with solvent. The final concentration of the spiking solution was about 8 μg/ml.

XP 280 stock spiking solution was prepared by accurately weighing 100 mg of XP 280 reference standard into a 100-ml volumetric flask, add about 50 ml of solvent, shaking to dissolve, and bring to volume with solvent (i.e., 25%, v/v, acetonitrile in water). The spiking solution was prepared by pipetting 10 ml of the stock solution into a 250-ml volumetric flask and bring to volume with solvent. The final concentration of the spiking solution was about 40 μg/ml.

Excipient blend was prepared by turbular mixing the appropriate amount of each excipient. For example, for the low magnesium stearate and low Explotab formulation, turbularly mix 0.24 g each of magnesium stearate and Explotab, and 119.52 g of lactose monohydrate. This gives 120 g of excipient blend, which is equivalent to 1000 capsule filled masses.

Sample solutions were prepared by transferring five capsule shells into an appropriate size container, weighing and transferring 600 mg of excipient blend (which is equivalent to five capsules) into the same container. The appropriate amounts of spiking solutions (25 to 100 ml, as indicated in Table 1) were then added. The solutions were then shaken for 30 min, allowed to stand for several minutes, and then filtered using 0.45-μm Whatman polyvinylidene difluoride (PVDF) HPLC filters. These were the sample solutions.

2.4. Chromatography methods

The HPLC system used in this study was a Hewlett-Packard 1100 with autosampler and a variable-wavelength UV detector.

For the DMP 543 method, a Zorbax RX C_{18}, 5 μm, 250×4.6 mm analytical column was thermostated at 40°C. The injection volume was 40 μl. The isocratic method used a flow-rate of 1.0 ml/min. Mobile phase was 60% (v/v) acetonitrile in 10 m*M* sodium phosphate, monobasic, solution. DMP 543 was detected at 260 nm. The method run time was 10 min.

For the DMP XP 280 method, a Zorbax RX C_{18}, 5 μm, 250×4.6 mm analytical column was thermostated at 40°C. The injection volume was 5 μl. The isocratic method used a flow-rate of 1.0 ml/min. Mobile phase was 25% acetonitrile in 75% trifluoroacetic acid (0.1%, v/v) solution. XP 280 was detected at 280 nm. The method run time was 7 min.

Table 1
Variables and their practical limits

Variable	Type	Limits
Drug substance	Categorical	XP 280 or DMP 543
Capsule shell size	Categorical	Size 3 or 0
Explotab	Continuous	0.2–10.0%
Magnesium stearate	Continuous	0.2–1.0%
Percentage of acetonitrile in sample solvent	Continuous	25–75%
Buffer concentration	Continuous	0–10 m*M*
Amount of sample solvent	Continuous	25–100 ml

2.5. ECHIP software

ECHIP software was purchased from ECHIP (Hockessin, DE, USA). The screening design uses the Plackett–Burman design with five replicates. The response surface design uses the central composite in cube design with five replicates. The software uses the results of the five replicates to determine assay error. All data analyses were done using ECHIP software.

3. Results and discussion

The systematic approach to determine, eliminate, and document assay bias involves four steps. The first step is to determine the variables that can affect assay results and define their practical lower and upper limits. The second step is to design and execute a screening experimental design to eliminate insignificant variables. The third step is to perform and execute a response surface design on significant variables to determine their primary, quadratic, and interaction effects. The last step is to optimize formulation and/or assay conditions under practical constraints.

3.1. Variables and their practical limits

There are two major sources of variables that can affect assay results, namely the formulation variables and the analytical method variables. Formulation variables include the nature of the drug substance, the size of the capsule shells, and the percentage of each excipient in the formulation. Analytical variables include percentage of organic in the sample solvent, the concentration of buffer in the sample solvent, and the amount of sample solvent used in sample preparation. The variables in this model study and their practical limits are given in Table 1.

3.2. Plackett–Burman screening design

All variables and their practical limits listed in Table 1 were entered into the ECHIP software. Categorical variables are the variables that can only have fixed values, e.g., size 0 or size 3 capsule shells. On the other hand, continuous variables can have any value between the lower and upper limits. A Plackett–Burman screening design with five replicates was generated by the software, and is given in Table 2. Experiments listed in Table 2 were carried out and the results are given in Table 3. The $\pm t(\sigma/\sqrt{n})$ range gives the 95% confidence interval and the

Table 2
Plackett–Burman (two-level) screening design with five replicates

Trial	Drug substance	Capsule size	Explotab (%)	Magnesium stearate (%)	Acetonitrile (%)[a]	Buffer (m*M*)[a]	Solvent (ml)
2	DMP 543	3	0.2	1.0	75.0	10.0	100.0
7	XP 280	0	0.2	1.0	25.0	0.0	100.0
6	XP 280	3	10.0	0.2	25.0	10.0	100.0
9	DMP 543	0	0.2	0.2	75.0	0.0	100.0
4	DMP 543	3	10.0	1.0	25.0	0.0	100.0
5	XP 280	3	0.2	1.0	75.0	10.0	25.0
11	XP 280	0	10.0	1.0	75.0	0.0	25.0
2	DMP 543	3	0.2	1.0	75.0	10.0	100.0
12	XP 280	3	0.2	0.2	25.0	0.0	25.0
8	DMP 543	0	0.2	0.2	25.0	10.0	25.0
10	DMP 543	3	10.0	0.2	75.0	0.0	25.0
3	XP 280	0	10.0	0.2	75.0	10.0	100.0
5	XP 280	3	0.2	1.0	75.0	10.0	25.0
4	DMP 543	3	10.0	1.0	25.0	0.0	100.0
3	XP 280	0	10.0	0.2	75.0	10.0	100.0
1	DMP 543	0	10.0	1.0	25.0	10.0	25.0
1	DMP 543	0	10.0	1.0	25.0	10.0	25.0

[a] In the sample solvent.

Table 3
Results of Plackett–Burman screening design with five replicates

Trial	Mean recovery (%)	$\pm t(\sigma/\sqrt{n})$ Range (%)	Bias (%)
2	100.7	100.5–100.9	+0.7
7	99.5	99.3–99.7	−0.5
6	98.9	98.7–99.1	−1.1
9	101.0	100.8–101.2	+1.0
4	99.4	99.2–99.6	−0.6
5	91.3	91.1–91.5	−8.7
11	83.8	83.6–84.0	−16.2
2	100.9	100.7–101.1	+0.9
12	98.0	97.8–98.2	−2.0
8	96.7	96.5–96.9	−3.3
10	101.8	101.6–102.0	+1.8
3	95.9	95.7–96.1	−4.1
5	91.2	91.0–91.4	−8.8
4	99.1	98.9–99.3	−0.9
3	95.6	95.4–95.8	−4.4
1	97.5	97.3–97.7	−2.5
1	97.3	97.1–97.5	−2.7

Replicate standard deviation is 0.16%. Student's t-value for 4 degrees of freedom ($n=5$) and 95% confidence limit is 2.776.

percentage of assay bias is defined as the difference between the percent mean recovery and 100%. Results were than entered into the software for data analysis. Qualitative results are captured by the Pareto effects graph given in Fig. 2. The Pareto effects graph provides an excellent snapshot of the effects of each variable. The vertical bars define the magnitude of the effects of each variable shown on the right side of the graph. Quantitative results are captured by the coefficients table, which is given in Table 4. The coefficient gives the unit effect of each variable. The constant term is the percentage of assay recovery, which would be 100% in the absence of assay bias. The P-value defines the significance of the measurement. A typical cut-off P-value for a statistically significant measurement is less than or equal to 0.05.

As shown in Table 2, the Plackett–Burman design is a well-balanced and randomized design. The design requires a total of 17 experiments; 12 different experiments and five repeats. The five repeats are used to determine the experimental error of the method and that in turn is used to determine the significance of the effects. The number of experiments required by the Plackett–Burman design is comparable to that required by the method of trial and error. The traditional trial and error method for a seven variable problem would require 14 experiments plus additional experiments used to determine method variation. The advantage of the experimental design approach is that it is much more informative and provides excellent documentation of the investigation.

It is almost impossible to recognize trends and assign causes by looking at the data from the results

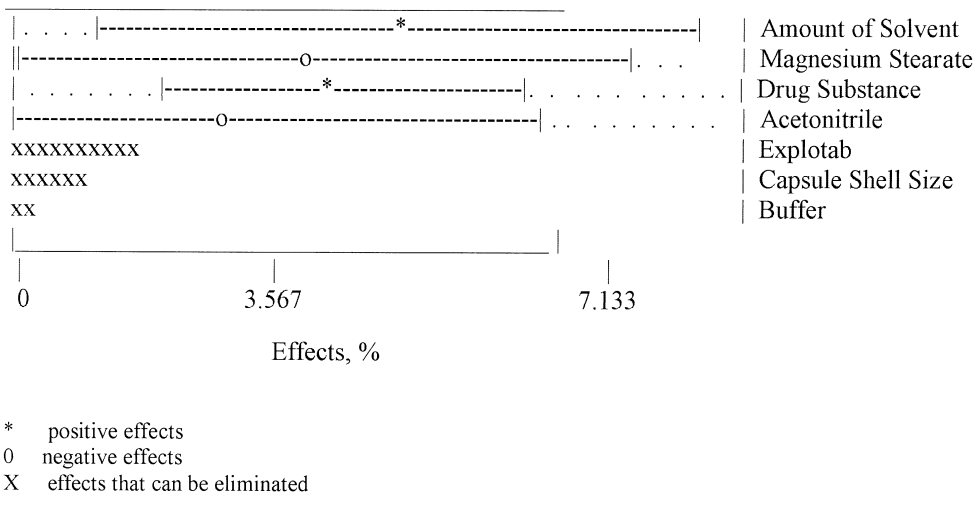


Fig. 2. Pareto effects graph on results from the Plackett–Burman screening design.

Table 4
Coefficients table of the Plackett–Burman screening design

Term	Coefficient	Standard deviation	*P*-value
Constant	97.056	N/A	N/A
Acetonitrile (%)	−0.050	0.029	0.1233
Magnesium stearate (%)	−0.029	0.306	0.0453
Buffer (m*M*)	0.027	0.145	0.8558
Amount of solvent	0.052	0.019	0.0218
Capsule shell size	−0.972	0.727	0.2136
Drug substance	2.50	0.724	0.0072
R^2=0.776, P=0.0209	Residual SD=2.879		
Adjusted R^2=0.602	Residual df=9		
	Replicate SD=0.16		
	Replicate df=5		
	Cross Val. RMS=5.977		

of the randomized experiments given in Table 3. However, it can be seen that the method gives negative assay bias under most conditions. In fact, trial 11 gives only 83.8% recovery. This is not due to experimental error. The standard deviation among the five replicate preparations of trials one to five is only 0.16%, which cannot account for the 16.2% negative assay bias. In addition, trial 11 was repeated and the results are the same. The five replicate trials used to determine the standard deviation of the method was chosen by the ECHIP software. As can be seen in Table 2, the software really does a nice job in choosing the five replicate experiments because those experiments contain extreme values for all variables.

Several possible causes of low recovery observed in trials 11 and 5 were ruled out experimentally. These include solution stability, solvent effects due to different solvents used in sample preparation and mobile phase, and adsorption of the drug by the HPLC filter. Solution stability was confirmed by re-injecting the same solution after several days; no decrease in assay value and no additional amount of degradation were observed. Performing analyses on standard solutions in the absence of excipients using different percentages of acetonitrile in the sample solvent eliminated solvent effects. Results are virtually identical for sample solvents up to 75% acetonitrile. An experiment was done on the same sample solution with and without filtering the sample solution prior to HPLC analysis. There was no difference in the results. The low assay results could be from a combination of variables, as discussed below.

The Pareto effects graph show which variables cause a positive, negative or no effect on the results. The Pareto effects graph in Fig. 2 shows that increasing the amount of solvent or going from XP 280 to DMP 543 drug substance have positive effects on assay results. On the other hand, increasing the amount of magnesium stearate in the formulation or the amount of acetonitrile in the sample solvent has negative effects on assay results. The magnitudes of these effects, for the whole practical limits, are displayed on the Pareto effects graph.

The Pareto effects graph explains the low assay results obtained from trials 5 and 11. Examining the variables of trials 5 and 11 (Table 2) reveals that these two trials are for XP 280, they contain high levels of magnesium stearate in the formulation and acetonitrile in sample solvent, and low amounts of sample solvent. The low recovery is due to a combination of these four variables. As indicated on the Pareto effects graph (Fig. 2), going from DMP 543 to XP 280 drug substance contributes a negative effect on the assay value (a positive effect would occur going from XP 280 to DMP 543). A high level of magnesium stearate in the formulation gives a negative effect as does a high level of acetonitrile in the sample solvent. Going to a low amount of sample solvent has a negative effect on assay values; a positive effect would occur going from low to high amounts of sample solvent.

The unit effects of each variable are given as coefficients in Table 4. Other important information given in Table 4 include the *P*-values of the effects from each variable, the replicate standard deviation of the method, and the R^2 value and the *P*-value associated with the model. The *P*-value is calculated based on the magnitude of the effects with respect to the replicate standard deviations. A *P*-value of ≤ 0.05 indicates that the variable is significant at the 0.05 level. Based on the *P*-values given in Table 4, it can be concluded that the amount of magnesium stearate in the formulation, the amount of solvent in sample preparation, and the drug substance itself are significant variables. Other variables are insignificant and can be eliminated from the model. In addition, the R^2 value shows that the model accounts for 77.6% of the experimental variations.

3.3. Central composite in cube response surface design

The screening design identified that the drug substance, the percentage of magnesium stearate in the formulation, and the amount of sample solvent as significant variables. A central composite in cube response surface design was generated by ECHIP, separately for DMP 543 and XP 280, to investigate primary, quadratic, and interaction effects from the significant variables. Note that the drug substance itself could also be treated as a variable and incorporated into the design, but this was not attempted in this study. A central composite in cube response surface design is a spherical design that covers each corner of the cube, the inside center point of the cube, and center points above each of the eight surface planes of the cube. The design is given in Table 5. Experiments listed in Table 5 were carried out for both DMP 543 and XP 280 drug products and the results are given in Table 6. Qualitative results are given in Figs. 3 and 4 and quantitative results are given in Tables 7 and 8.

Table 5
Central composite in cube response surface design with five replicates

Trial	Magnesium stearate (%)	Amount of sample solvent (ml)
4	1.00	100.0
11	0.47	75.0
9	1.00	50.0
6	0.20	25.0
3	0.60	100.0
8	0.47	25.0
2	0.20	62.5
4	1.00	100.0
1	0.20	100.0
5	1.00	25.0
10	1.00	75.0
1	0.20	100.0
5	1.00	25.0
7	0.73	25.0
2	0.20	62.5
3	0.60	100.0

Table 6 shows that, in general, there is a positive assay bias in the DMP 543 method and a negative assay bias in the XP 280 method under most conditions. This is consistent with the trends observed from the screening design. The Pareto effects graphs, Figs. 3 and 4, show that the amount of sample solvent negatively affects DMP 543 assay results but positively affects XP 280 results. In addition, Fig. 4 shows that the XP 280 method may have an additional primary effect from the amount of magnesium stearate in the formulation, an interaction effect from magnesium stearate and the amount of sample solvent, and a quadratic effect from the amount of sample solvent. However, the *P*-values given in Tables 7 and 8 show that the variables in the DMP 543 method are not significant at the $P=0.05$ level. Only the amount of sample solvent is significant at the $P=0.05$ level in the XP 280 method. The other three additional effects for XP 280 method mentioned above and displayed in Fig. 4 are only significant at the $P=0.15$ level. In addition, the R^2 value given in Tables 7 and 8 show that the model only explains 42.7% of the variation in the DMP 543 method but explains 90.8% of the data in the XP 280 method. This can also be seen from the plot of observed value versus fitted value, which is given in Fig. 5. The observed values are the experimental results and the fitted values are the predicted values by the central composite in cube response surface design model. The low R^2 value for DMP 543 results is due to low assay variation.

The results of the response surface design experiments of the DMP 543 assay method concluded that none of the factors had a significant effect on assay

Table 6
Results of central composite in cube response surface design with five replicates

Trial	DMP 543			XP 280		
	Mean recovery (%)	$\pm t(\sigma/n^2)$ Range (%)	Bias (%)	Mean recovery (%)	$\pm t(\sigma/n^2)$ Range (%)	Bias (%)
4	100.2	99.8–100.6	No bias	100.2	99.8–100.6	No bias
11	100.2	99.8–100.6	No bias	99.9	99.5–100.3	No bias
9	100.2	99.8–100.6	No bias	99.9	99.5–100.3	No bias
6	101.0	100.6–101.4	+1.0	98.4	98.0–98.8	−1.6
3	100.9	100.5–101.3	+0.9	100.3	99.9–100.7	No bias
8	101.8	101.4–102.2	+1.8	98.4	98.0–98.8	−1.6
2	100.9	100.5–101.3	+0.9	99.5	99.1–99.9	−0.5
4	100.8	100.4–101.2	+0.8	100.3	99.9–100.7	No bias
1	100.9	100.5–101.3	+0.9	100.2	99.8–100.6	No bias
5	101.9	101.5–102.3	+1.9	98.9	98.5–99.3	−1.1
10	101.2	100.8–101.6	+1.2	100.1	99.7–100.5	No bias
1	100.5	100.1–100.9	+0.5	100.5	100.1–100.9	+0.5
5	101.3	100.9–101.7	+1.3	99.0	98.6–99.4	−1.0
7	101.3	100.9–101.7	+1.3	99.0	98.6–99.4	−1.0
2	100.5	100.1–100.9	+0.5	99.9	99.5–100.3	No bias
3	100.5	100.1–100.9	+0.5	101.2	100.8–101.6	+1.2

Replicate standard deviations are 0.35 and 0.33%, respectively for DMP 543 and XP 280. Student's *t*-value for 4 degrees of freedom ($n=5$) and 95% confidence limit is 2.776.

result. However, the amount of sample solvent, and possibly the amount of magnesium stearate ($P=0.11$) affect XP 280 assay results, therefore these variables need to be optimized.

3.4. Optimization of assay conditions

In theory, both the amount of magnesium stearate in the formulation and the amount of sample solvent used in the assay can be optimized simultaneously or separately. In reality, however, it is more practical and easier to optimize the analytical method conditions. The ECHIP software can predict the optimal conditions. Fig. 6 shows that by fixing the amount of magnesium stearate at 0.75% (4.5 mg) that a 100% recovery can be achieved by using 60 ml of sample solvent in the method. The prediction was confirmed experimentally.

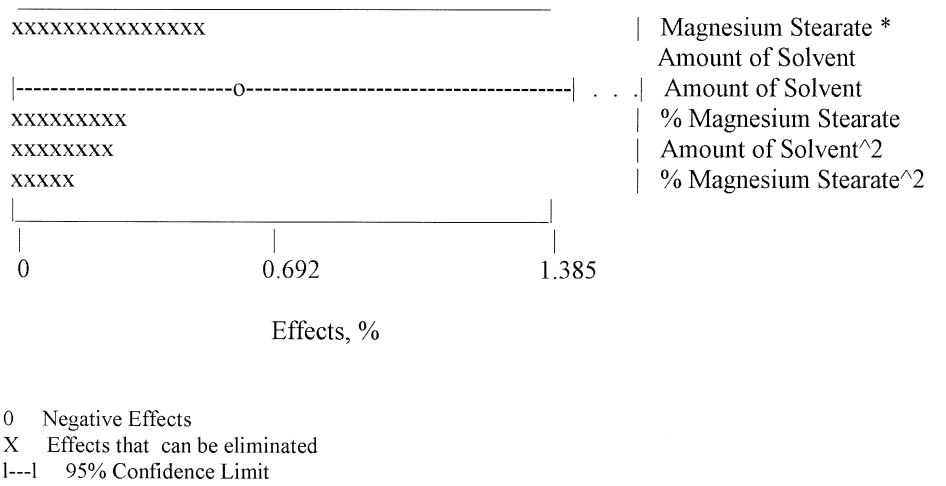
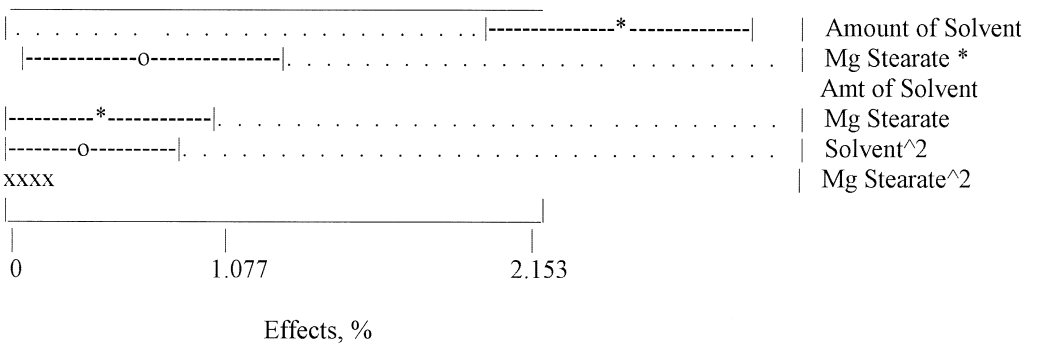


Fig. 3. Pareto effects graph of DMP 543 on results from the central composite in cube response surface design.



* Positive Effects
0 Negative Effects
X Effects that can be eliminated
l---l 95% Confidence Limit

Fig. 4. Pareto effects graph of XP 280 on results from the central composite in cube response surface design.

Table 7
Coefficients table of the central composite in cube response surface design for DMP 543

Term	Coefficient	SD	*P*-value
Constant	100.75	N/A	N/A
Magnesium stearate (%)	0.064	0.066	0.3570
Amount of solvent	−0.007	0.004	0.1409
Magnesium stearate*amount of solvent	−0.003	0.002	0.2192
(Magnesium stearate)^2	−0.029	0.054	0.6020
(Amount of solvent)^2	0.000	0.000	0.4172
R^2=0.427, P=0.2763	Residual SD=0.521		
Adjusted R^2=0.140	Residual df=10		
	Replicate SD=0.35		
	Replicate df=5		
	Cross Val. RMS=0.789		

Table 8
Coefficients table of the central composite in cube response surface design for XP 280

Term	Coefficient	SD	*P*-value
Constant	99.98	N/A	N/A
Magnesium stearate	0.067	0.038	0.1058
Amount of solvent	0.023	0.002	0.0000
Magnesium stearate*amount of solvent	−0.002	0.001	0.0991
(Magnesium stearate)^2	−0.031	0.030	0.3313
(Amount of solvent)^2	−0.000	0.000	0.1164
R^2=0.908, P=0.0001	Residual SD=0.295		
Adjusted R^2=0.861	Residual df=10		
	Replicate SD=0.33		
	Replicate df=5		
	Cross Val. RMS=0.265		

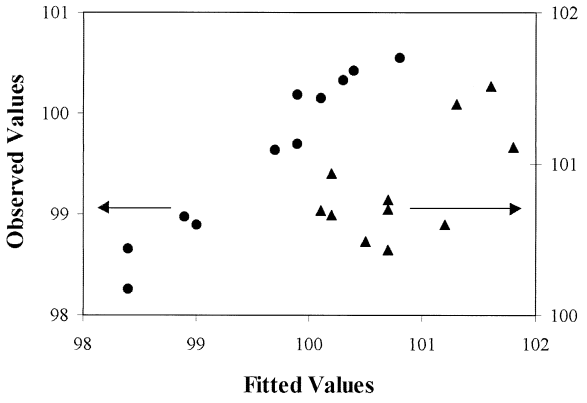


Fig. 5. Plot of observed values versus fitted values. (●)=XP 280 and (▲)=DMP 543.

4. Conclusions

It has been shown from these model studies that experimental design is a powerful tool to systematically investigate and document assay bias. Even though there is no regulatory guideline that states what is an acceptable level of assay bias, it is a good practice to investigate variables that can impact assay results. Results from the assay bias investigation provide useful information on the robustness of the formulation and the analytical method. This information in turn may prevent disasters during late developmental stages.

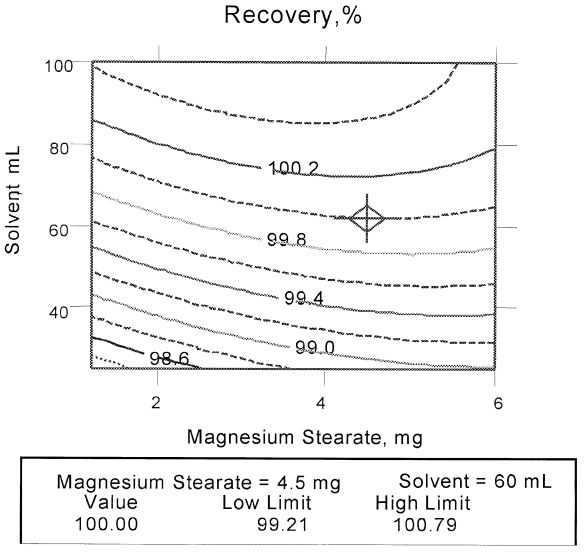


Fig. 6. Contour plot of optimization map generated by ECHIP.

Acknowledgements

The authors wish to thank Mary Ann Gorko for her inputs on experimental design and interpretation of statistics and thank Dr. David K. Lloyd for his input and helpful discussions. Special thanks go to DuPont Pharmaceuticals Summer Internship Program for its financial support.

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