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Validation of a dissolution method with HPLC analysis for lasofoxifene tartrate low dose tablets

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Abstract

A dissolution method with high performance liquid chromatography (HPLC) analysis was validated for an immediate release low dose tablet formulation. The method was validated to meet requirements for a global regulatory filing and this validation included specificity, precision, linearity, accuracy and range. Validation of precision included an intermediate precision study using an experimental design in order to satisfy Japanese regulatory requirements. In addition, filter suitability, standard and sample solution stability and method robustness were demonstrated. A statistical design of experiments was used for the robustness evaluation of both the dissolution method and the HPLC analysis method. All results were acceptable and confirmed that the method is suitable for its intended use.

Keywords: Dissolution; Validation; Intermediate precision; Statistical design; HPLC; Method robustness; Low-dose tablet

1. Introduction

Dissolution testing is used to guide development of new drug products and to assess lot-to-lot variability of drug products. Dissolution methods, as well as other analytical methods, are validated to ensure they are suitable for their intended use and give accurate and reliable data. Guidances on validation characteristics and considerations have been published [1,2]. Validation of a dissolution method typically involves validation of the end analysis method for specificity, precision, linearity, accuracy and range.

There are three categories of precision—repeatability, reproducibility and intermediate precision. Repeatability is the precision of the method under the same operating conditions over a short period of time. Reproducibility determines the precision between laboratories. Intermediate precision is a measure of intra-laboratory variance using different operators on different days, equipment, etc. and is not required in cases where reproducibility has been performed [1]. For Japanese regulatory authorities, however, intermediate precision is considered a necessity [3]. Therefore, in the validation of this analytical procedure, intermediate precision was performed using an experimental design in order to fully satisfy Japanese regulatory requirements [4].

Method robustness is an additional characteristic of the method that should be studied. According to ICH Q2A, robustness of a method is defined as "a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage [1]." Method robustness should be evaluated and if measurements are affected by variations in method parameters, then these should be controlled or a statement should be included in the method [2]. A number of papers have been published detailing robustness studies for the dissolution end analysis method [5–8], while only a few papers have been published describing method robustness studies for the dissolution portion of the method [9,10].

This paper describes the validation of a dissolution method for 0.25 and 0.5 mg lasofoxifene tartrate immediate release tablets. Lasofoxifene tartrate, shown in Fig. 1, is an estrogen agonist/antagonist under development for the prevention and treatment of osteoporosis [11,12]. In addition, filter suitability,

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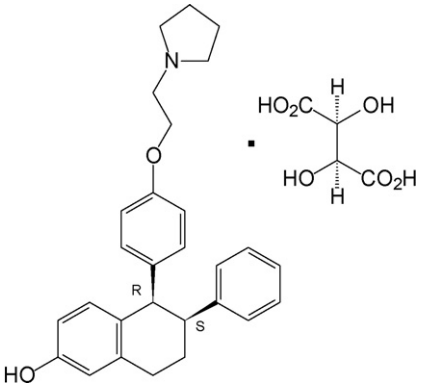

Fig. 1. Structure of lasofoxifene tartrate.

standard and sample solution stability and method robustness were demonstrated. A statistical design of experiments was used for the robustness evaluation of both the dissolution method and the high performance liquid chromatography (HPLC) analysis method.

2. Experimental

2.1. Materials

Concentrated (28–30%) ammonium hydroxide, concentrated hydrochloric acid, both reagent grade, and acetonitrile (HPLC grade) were obtained from J.T. Baker (Phillipsburg, NJ, USA). Trifluoroacetic acid (HPLC grade) was obtained from Pierce (Rockford, IL, USA). Purified water for dissolution and chromatography were obtained from a Milli-Q purification unit (Millipore, Milford, MA, USA). Disposable latex-free 10 mL plastic syringes were supplied by Henke Sass Wolf (Germany), Acrodisk 25 mm syringe filters with 1 μm Glass Fiber membrane by Pall (East Hills, NY, USA) and cannulae made from teflon tubing by Upchurch (Oak Harbor, WA, USA).

2.2. Instrumentation

For all dissolution experiments, a DISTEK 2100A or DISTEK 2100C (N. Brunswick, NJ, USA) were used. For preparation of standards and mobile phase an analytical balance (Mettler-Toledo AG245; Switzerland) and pH meter (VWR 2100; West Chester, PA, USA) were used. For all post-dissolution analyses an Agilent HP1100 (Agilent; Palo Alto, CA, USA) was used. Data acquisition was performed using Turbo*Chrom (Perkin-Elmer; Wellesley, MA, USA).

2.3. Sampling device

Two different sampling devices were used for the dissolution experiment.

Sampling device A consisted of the following: teflon tubing (1/16 in. i.d. × 1/8 in. o.d.), cut into 6 in. segments from longer teflon tubing (e.g., Upchurch Part #1521 for 20 ft or Upchurch Part #1523 for 10 ft) and luer-lock adapters (e.g., Upchurch Part #P-857X) to connect the 6 in. sampling tubing to syringes.

Sampling device B consisted of the following: a 1/4–28, tefzel, female luer to female adapter (Upchurch Part #P-628), a bent 6 in. long, 1/8 in. o.d. teflon tubing, a 1/8 in. tefzel, flangeless ferrule (Upchurch Part #P-300NX) and a 1/8 in., 1/4–28, PFA, flangeless nut (Upchurch Part #P-345X).

2.4. Methods

2.4.1. Dissolution test conditions

Dissolution testing was performed in compliance with USP ⟨711⟩ using apparatus 2 with paddles. A dissolution media/agitation screen was performed for medium and paddle speed selection. A dissolution medium of 0.01N HCl was chosen based on the profiles obtained and its environmental benefits compared to 0.1N HCl. A paddle speed of 75 rpm was selected to minimize coning. The media volume used was 500 mL. The medium, which was vacuum degassed under house vacuum, was maintained at 37 ± 0.5 °C. The 1-L glass dissolution vessels were covered to minimize evaporation. Samples were drawn at 15, 30, 45 and 60 min for early validation work. Later, as acceptance criteria were altered, samples were drawn at 5, 10, 20 and 30 min. As lasofoxifene tartrate tablets are immediate releasing, the earlier timepoints provided more discriminating ability. Manual sampling was performed using 10 mL aliquots. These solutions were immediately filtered using an Acrodisk 25 mm syringe filters with 1 μm Glass Fiber membrane. The first 7 mL of sample was discarded prior to collecting the sample for analysis.

2.4.2. HPLC method

An HPLC method with UV detection was selected for the method of analysis. The reversed-phase procedure utilized a Waters Symmetry C_{18} column (3.5 μm; 50 mm × 2.1 mm i.d.) and UV detection at 206 nm. This wavelength was selected because it is a UV maximum and provides the sensitivity needed for quantitation of the low drug concentration in the dissolution samples. The column temperature was maintained at 40 °C. The mobile phase contained purified water, acetonitrile, trifluoroacetic acid and ammonium hydroxide (65:35:0.25:0.2, v/v/v/v, respectively). The flow rate was 0.5 mL/min for 6 min with an injection volume of 100 μL. A standard solution of active pharmaceutical ingredient (API) was prepared first in mobile phase, and subsequently diluted down to the appropriate concentration with dissolution medium. This standard solution contained 100% of the final assay concentration of drug (∼0.5 μg/mL).

2.5. Statistical analysis

Statistical analyses and graphical enhancements of designed experiments were performed using Design Expert v6.05 and SAS v8.2.

3. Results and discussion

3.1. Validation of the HPLC method

The HPLC method used to analyze the lasofoxifene tartrate tablet dissolution samples was validated according to current ICH guidelines. Validation included specificity, precision, linearity (in the presence and absence of excipients) and range. In addition, filter suitability, solution stability and robustness were evaluated. Samples were drawn at 15, 30, 45 and 60 min for early validation work. Later, as acceptance criteria were altered, samples were taken at 5, 10, 20 and 30 min.

At the time of this validation the following sample preparation approaches were considered: (1) preparing a composite sample by grinding multiple tablets together; (2) preparing a solution of drug and spiking it into a dry excipient blend or (3) weighing drug into a dry excipient blend. Since this was a high potency compound, sample preparations involving lasofoxifene tartrate were handled in a containment isolator that made sample preparation difficult. Therefore, the first and last options were not desirable and a solution of drug spiked into a dry excipient blend was selected as the best choice to minimize sample-handling issues. In addition, whereas the sample prepared in the presence of excipients was stable for only 2 days, option two had the advantage of the stock drug solution being stable for 30 days.

3.1.1. Specificity

Specificity was examined by analyzing a solution of a placebo containing the excipient mixture for the lasofoxifene tartrate tablet dissolved in the dissolution medium. Absence of interference was demonstrated.

3.1.2. Precision

Precision must be evaluated on a reliable, homogeneous test sample. If a homogeneous sample is not readily available, a prepared test sample that is presumed to be homogeneous can be used [13]. Synthetic blend solutions representing 100% of the target concentration of the method (0.5 μg lasofoxifene/mL) were used. A representative synthetic blend approach was utilized in order to minimize any potential inconsistencies due to variations in tablet content uniformity.

Precision was evaluated by performing repeatability (instrument and method precision), intermediate precision and reproducibility.

3.1.3. Instrument precision

The injection precision of the method was evaluated by performing six replicate injections of a sample at the nominal 0.25 mg standard concentration (~0.5 μg lasofoxifene/mL). The sample was a synthetic blend of drug and excipients. The peak area R.S.D. (%) was 0.3% which was considered acceptable.

3.1.4. Method precision

The R.S.D. (%) of the sample response factor was calculated for six separate preparations at the nominal standard concentration of the 0.25 and 0.5 mg tablets (~0.5 μg lasofoxifene/mL). The sample was a synthetic blend of drug and excipients. The peak area R.S.D. (%) values were 4.3 and 1.9%, which were considered acceptable for these low dose drug product formulations.

Table 1
Intermediate precision results

Test day	Analyst	Instrument	Column	Number of replicates	Drug (%)
1	A	A	A	2	99.0, 98.7
2	A	B	A	2	101.3, 100.2
3	A	A	B	2	99.8, 100.3
4	B	B	A	2	100.5, 100.4
5	B	A	B	2	98.5, 101.3
6	B	B	B	2	97.6, 97.5
Mean, R.S.D. (%)					99.6, 1.3

3.1.5. Intermediate precision

Intermediate precision was performed by two analysts, each testing two sample preparations on three separate days and using two different instruments and analytical columns. Fresh sample and standard solutions were independently prepared on each day of analysis. The intermediate precision results are shown in Table 1. These results were considered acceptable.

3.1.6. Reproducibility

As shown in Table 2, acceptable interlaboratory reproducibility of the dissolution method was demonstrated through the successful transfer of the method from the development laboratory to a contract testing facility. The transfer consisted of the analysis of 12 tablets.

Table 2
Reproducibility results

Sampling time	
15 min	
Mean dissolved (%)	98
R.S.D. (%)	2.7
Mean comparative data[a]	96
Difference from comparative data[b] (%)	2
30 min	
Mean dissolved (%)	101
R.S.D. (%)	1.5
Mean comparative data[a]	98
Difference from comparative data[b] (%)	3
45 min	
Mean dissolved (%)	102
R.S.D. (%)	1.7
Mean comparative data[a]	98
Difference from comparative data[b] (%)	4
60 min	
Mean dissolved (%)	100
R.S.D. (%)	2.3
Mean comparative data[a]	98
Difference from comparative data[b] (%)	2

[a] Data obtained by the transferring laboratory.
[b] Calculated on an absolute basis by subtracting the mean obtained at the receiving laboratory from the mean obtained in the laboratory.

Table 3
Percent recoveries and relative standard deviations at the 0.25 and 0.5 mg level

0.25 mg Tablets				0.5 mg Tablets			
Theoretical spiked level (μg lasofoxifene/mL)	Average recovery (%)	Range of recoveries	R.S.D. (%)	Theoretical spiked level (μg lasofoxifene/mL)	Average recovery (%)	Range of recoveries	R.S.D. (%)
0.2569	98	96–100	2.5	0.5138	98	97–100	1.9
0.5138	99	92–102	5.1	1.0276	98	96–101	0.9
0.6423	98	97–100	1.5	1.2845	98	97–100	1.5

Table 4
Standard and sample solution stability results

Sample	Concentration (μg/mL)	Percentage of initial at 7 days	Percentage of initial at 22/23 days[a]	Percentage of initial at 42/43 days[a]
0.25 mg sample	0.5	96	NT	NT
0.5 mg sample	1.0	99	NT	NT
0.25 mg working standard	0.5	99	98	NT
0.5 mg working standard	1.0	101	100	NT
Stock standard	0.5	101	102	99

NT: not tested.
[a] 22 and 23 days for the 0.25 and 0.5 mg working standards, respectively; 42 and 43 days for the 0.25 and 0.5 mg stock standard, respectively.

3.1.7. Linearity

The linearity of the lasofoxifene response was evaluated from 25% of the lowest concentration (0.13 μg lasofoxifene/mL) to 125% of the highest concentration (1.3 μg lasofoxifene/mL). Individual samples were prepared at each concentration level from one stock solution.

These data indicate that the lasofoxifene peak area is linear over the concentration range of 0.13–1.3 μg lasofoxifene/mL. The R^2 for the regression line is 0.9999 with a slope of 4.662×10^5 and a *y*-intercept of −2164.3. The *y*-intercept is within 10% of the response at the 100% level. The residual sum of squares is 14,134,791.0. These results were considered acceptable.

3.1.8. Linearity in presence of excipients

The linearity of the drug response in the presence of excipients was evaluated from 25% of the lowest concentration (0.13 μg lasofoxifene/mL) to 125% of the highest concentration (1.3 μg lasofoxifene/mL). Individual samples were prepared at each concentration level from one stock solution of drug spiked into excipients.

These data indicate that the lasofoxifene peak area is linear over the concentration range of 0.13–1.3 μg lasofoxifene/mL in the presence of excipients using the chromatographic parameters described in the test procedure. The R^2 for the regression line is 0.9999 with a slope of 4.623×10^5 and a *y*-intercept of 673.5. The *y*-intercept is within 10% of the response at the 100% level. The residual sum of squares is 1,108,885.3. These results were considered acceptable.

3.1.9. Accuracy

Samples at 50, 100 and 125% of the nominal assay concentration were prepared for accuracy testing of 0.25 and 0.5 mg tablets. Each concentration was prepared in triplicate, except at the nominal concentration where six preparations were made, and each solution was injected once. The samples were prepared by weighing out a synthetic blend of drug and excipients. Recovery of lasofoxifene was determined for each sample through comparison of the response factors to that of a working standard prepared at the intended concentration (0.5 and 1.0 μg lasofoxifene/mL). The theoretical concentration is calculated by assuming exactly 35 mg of lasofoxifene tartrate was weighed, and then multiplying that number by the potency factor and dividing by the appropriate dilution factor. Results are shown in Table 3.

The data demonstrate full recovery of lasofoxifene over the range of 50–125% (0.5138–1.2845 μg lasofoxifene/mL) of the nominal concentration for both the 0.25 and 0.5 mg levels. The percent recoveries and percent relative standard deviations were considered acceptable.

3.1.10. Standard and sample solution stability

The stability of stock standard solutions, working standard solutions and samples was determined. The stock standards were prepared in mobile phase and subsequently diluted with dissolution media to arrive at the working standards. The solutions were stored under normal laboratory conditions (capped in flask at ambient temperature, unprotected from light). Recovery was determined using an external standard prepared on the day of analysis and comparing the result to the initial time-point. Stock standards were found to be stable for up to 42 days. Results are shown in Table 4.

Table 5
Factors for the robustness study

	Low	Nominal	High
pH	2.8	3.0	3.2
Flow rate (mL/min)	0.4	0.5	0.6
Temperature (°C)	35	40	45
Organic (%)	33	35	37

Table 6
Randomized split plot robustness design for the HPLC end analysis method and results

Randomized robustness design				Chromatographic setup				Acceptance criteria		
Temperature	pH	Flow rate	Organic (acetonitrile) (%)	Temperature (°C)	pH	Flow rate (mL/min)	Organic (acetonitrile) (%)	Retention time ≥2.5 and ≤4.5 (min)	Peak efficiency ≥1000	Peak asymmetry ≤2.0
+	−	−	−	45	2.8	0.4	33	5.6	5180	1.1
+	−	+	+	45	3.2	0.6	33	3.7	4879	1.1
+	+	−	−	45	2.8	0.4	37	3.1	5290	1.0
+	+	+	+	45	3.2	0.6	37	2.2	4612	1.1
−	−	−	−	35	2.8	0.4	33	6.5	5640	1.0
−	+	−	−	35	2.8	0.4	37	3.5	5117	1.1
−	−	+	+	35	3.2	0.6	33	4.4	4780	1.0
−	+	+	+	35	3.2	0.6	37	2.5	4262	1.1
+	+	−	+	45	2.8	0.6	37	2.1	4579	1.1
+	−	−	+	45	2.8	0.6	33	3.7	4691	1.1
+	−	+	−	45	3.2	0.4	33	5.7	5506	1.1
+	+	+	−	45	3.2	0.4	37	3.3	5542	1.1
−	−	+	−	35	3.2	0.4	33	6.6	5029	1.1
−	+	+	−	35	3.2	0.4	37	3.6	5192	1.1
−	−	−	+	35	2.8	0.6	33	4.4	4667	1.0
−	+	−	+	35	2.8	0.6	37	2.4	4453	1.1

"+" Refers to the high values in the chromatographic setup. "−" Refers to the low values in the chromatographic setup.

3.1.11. Range

Based on linearity, accuracy and precision data, the validated range of the method is from 50% of the lowest nominal concentration (0.26 μg lasofoxifene/mL) to 125% of the highest nominal concentration (1.28 μg lasofoxifene/mL) of lasofoxifene.

3.1.12. Robustness of HPLC end analysis

A statistical design of experiments was used to evaluate the robustness of the HPLC end analysis method. The variables evaluated in the study are shown in Table 5 and include pH, flow rate, column temperature, and %organic in the mobile phase. The parameters and criteria used to define robustness were retention time (>2.5 and ≤4.5 min), peak asymmetry (≤2.0) and efficiency (>1000).

The randomized robustness design in tabular form is illustrated in Table 6 along with the results of the study. Analysis of variance (ANOVA) techniques and regression analysis combined with graphical illustrations were used to determine the impact of the four variables of interest.

The most important factors that affect peak asymmetry are %organic, flow rate and temperature. The method, however, is robust with respect to peak asymmetry; this value was consistently <1.2 across all values of these parameters. Method suitability, therefore, will not be adversely affected with respect to peak asymmetry.

Flow rate is the parameter that most significantly affects peak efficiency. The method, however, is robust with respect to peak efficiency; this value was consistently >4200 across all values of these parameters. Method suitability, therefore, will not be adversely affected with respect to peak efficiency.

Changes in %organic and flow rate can impact retention time as shown in Fig. 2. Retention time decreases with increasing %organic and increasing flow rate.

The intent of the robustness study is to find a set of values for temperature, %organic, flow rate and pH that meet all method suitability criteria such that small to moderate deviations from these values will not have a significant adverse effect measured against these criteria. As shown in Fig. 3, method suitability criteria are met and the most robust conditions exist if the parameters are set such that %organic is 36%, flow rate is 0.50 mL/min, temperature is 40 °C, and pH is 3.00. The method suitability region shifts as %organic is increased. With temperature controlled at 40 ± 2 °C and flow rate controlled at 0.50 ± 0.05 mL/min, %organic must be controlled at 36 ± 1% to

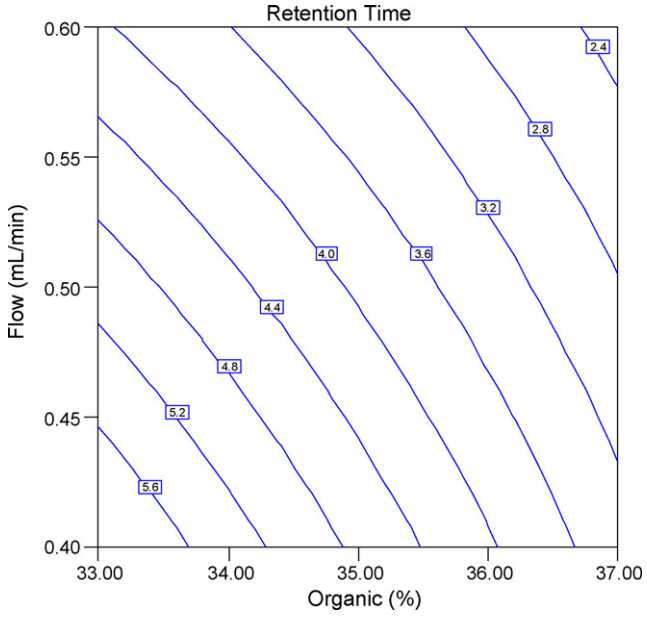

Fig. 2. Contour plot showing the effect of flow rate and %organic on retention time (temperature = 40 °C and mobile phase pH 3.0).

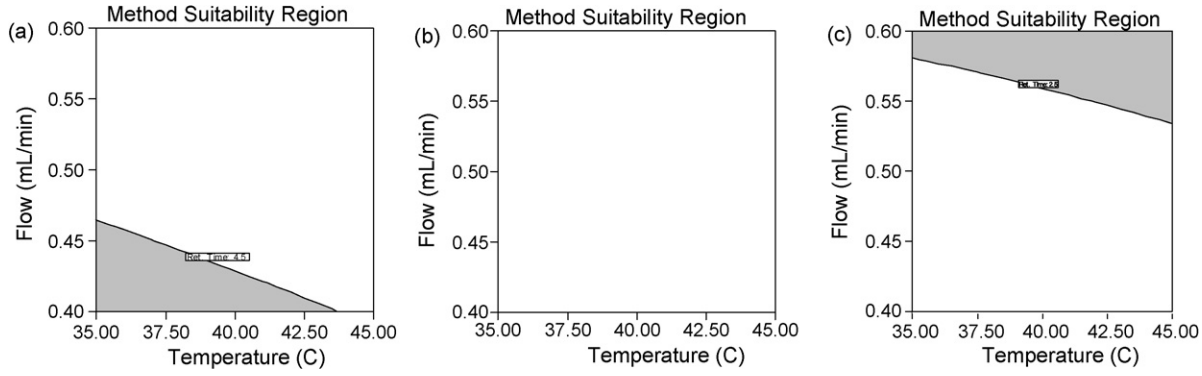

Fig. 3. Method suitability region for: (a) %organic = 35, pH 3.0; (b) %organic = 36, pH 3.0; and (c) %organic = 37, pH 3.0. The white region represents the suitable area.

Table 7
Robustness of dissolution variables

Parameter	Low	Nominal	High
Paddle	Teflon coated (TC)	Teflon coated (TC)	Solid teflon (ST)
Sampling device	A	B	B
Degas procedure	Overnight: house vacuum (HV)	15 min degas (15 min)	Overnight: open and stirring (OS)

meet the retention time method suitability requirement criteria ($2.5 \leq$ retention time ≤ 4.5). The mobile phase pH, in the range studied in this robustness experiment, has a negligible affect on suitability criteria.

Robustness for the method is, therefore, ensured by controlling the column temperature within $\pm 2\,^{\circ}C$ of target ($40\,^{\circ}C$), controlling the flow rate at 0.5 mL/min ± 10% and controlling the acetonitrile content in the mobile phase at 35–37%.

3.2. Validation of the dissolution method

3.2.1. Filter suitability

Two filters (Whatman GD/X 2.7 μm Glass Microfiber and Acrodisk 1 μm Glass Fiber) were evaluated using the dissolution medium and the following: (1) a solution containing lasofoxifene tartrate and excipients at 10% of the nominal concentration, (2) a solution containing lasofoxifene tartrate without excipients at 10% of the nominal concentration, (3) a solution containing lasofoxifene tartrate and excipients at 100% of the nominal concentration and (4) a solution containing lasofoxifene tartrate without excipients at 100% of the nominal concentration. The above solutions were prepared in a dissolution vessel and stirred at 75 rpm at $37\,^{\circ}C$ for 60 min. Samples were filtered and fractions collected at 1, 2, 3, 5, 7 and 10 mL. A small, potentially, interfering peak was observed in samples filtered through the Whatman filter. There were no interfering peaks observed in samples filtered with the Acrodisk. The Acrodisk 1 μm Glass Fiber filter showed recov-

Table 8
Randomized validation of dissolution experiment

Dissolution run	Factor combination	Paddle	Sampling device	Degas procedure
1	1	TC	A	HV
2	1	TC	A	HV
3	2	ST	B	OS
4	2	ST	B	OS
5	3	ST	B	HV
6	3	ST	B	HV
7	4	TC	A	OS
8	4	TC	A	OS
9	Nominal	TC	B	15 min degas
10	Nominal	TC	B	15 min degas
11	5	ST	A	HV
12	5	ST	A	HV
13	6	TC	B	HV
14	6	TC	B	HV
15	7	ST	A	OS
16	7	ST	A	OS
17	8	TC	B	OS
18	8	TC	B	OS

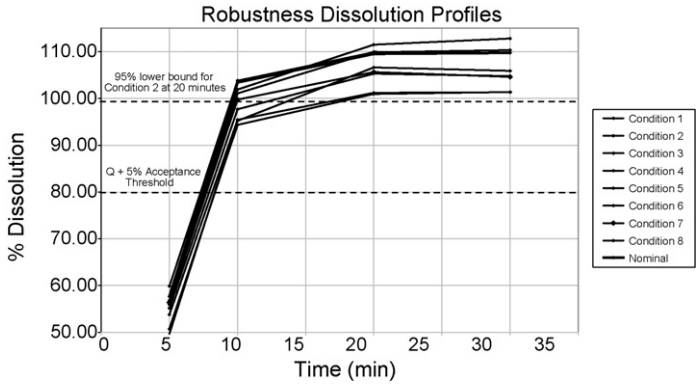

Fig. 4. Dissolution profiles from the robustness design.

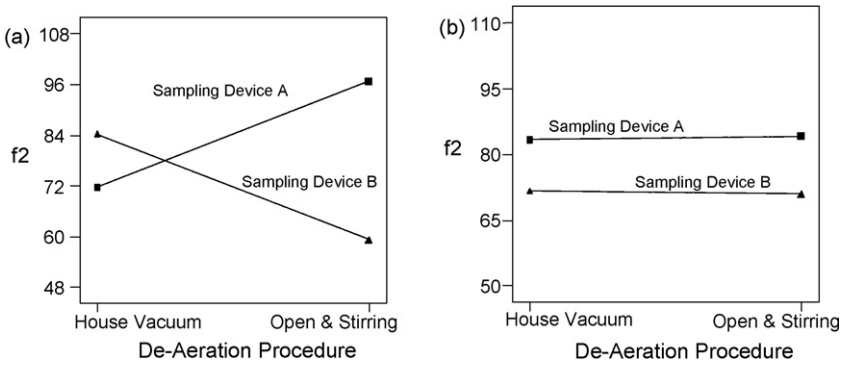

Fig. 5. Sampling device and degas procedure interactions for: (a) teflon coated paddles and (b) solid teflon paddles.

eries between 98 and 103% after discarding the first 7 mL of filtrate.

3.2.2. Robustness

A statistical design of experiments was utilized to evaluate the robustness of the dissolution test. The variables studied are shown in Table 7 and include paddle type, sampling device and degassing procedure. These variables were chosen as they were deemed the most significant factors that can potentially affect dissolution results. The degassing procedure parameter involved three conditions. The media was either left spinning in an open flask overnight, left vacuum degassing overnight, or vacuum degassed for 15 min as per the method. The first two options were chosen to maximize and minimize, respectively, the amount of dissolved oxygen in the media. The response of interest is %lasofoxifene released and method robustness was judged by f_2-similarity using only the 5 and 10 min timepoints since greater than 85% drug is dissolved at 10 min. The Model Independent Multivariate Confidence Region Procedure for judging robustness was not necessary since the within experimental-condition R.S.D. (%) was less than 15%, and a Model Dependent Approach was inappropriate since no mathematical model adequately fit the data [14].

A three parameter experimental design was set up using $2^3 = 8$ unique conditions (factor combinations) that allowed for the robustness verification. The experimental procedure was set up as a completely randomized design, which allowed for unbiased data-based conclusions to be made. The actual randomized experiment is shown in Table 8. Analysis of variance techniques and regression analysis combined with graphical illustrations were used to determine the impact of the variables under investigation. Fig. 4 shows the actual dissolution profiles for the eight design conditions and the nominal condition. For a given condition, the %dissolution at each time-point is the mean %dissolution for 12 vessels. Acceptance criteria (at 20 min % dissolved must be $\geq Q + 5\%$, where $Q = 75\%$) is clearly met; as shown in Fig. 4, the 95% lower bound on the true mean %dissolution rate at 20 min for condition 2 is 99.5%, well above the 80% threshold. The dissolution method is, therefore, considered robust for all parameters and conditions studied.

As shown in Fig. 5, for teflon coated paddles, if the media is de-aerated via house vacuum, the f_2 value is higher for sampling device B. If the media is de-aerated in a flask that is left uncapped and stirring over night, then the f_2 value is higher for sampling device A. This interaction between de-aeration method and sampling device is only evident when the teflon coated paddle is used; if the solid teflon paddle is used f_2 is higher for sampling device A regardless of which de-aeration method is employed. These marginally statistically significant results, however, are of little practical consequence since f_2 values were >61 across all experimental conditions.

4. Conclusions

A dissolution method with HPLC analysis for lasofoxifene tartrate low dose tablets has been fully validated to meet global

regulatory requirements. The methodology was evaluated for specificity, linearity, precision, accuracy and range in order to establish the suitability of the analytical method. Robustness of the dissolution method as well as robustness of the HPLC end analysis method was evaluated using statistical experimental designs. In addition, intermediate precision to satisfy Japanese regulatory requirements was performed and showed that there were no significant differences among the different "intermediate conditions" evaluated.

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