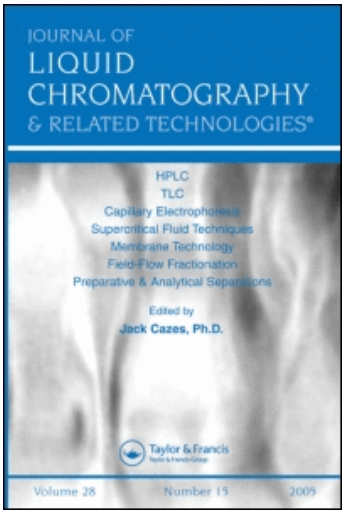

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USE OF EXPERIMENTAL DESIGN IN THE OPTIMIZATION OF HPLC METHODOLOGY FOR THE SEPARATION OF STEREOISOMERS

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ABSTRACT

Statistical experimental design was used to optimize chromatographic separations of two pharmaceutical compounds from their respective stereoisomeric impurities. The HPLC separations employ beta-cyclodextrin as a mobile phase additive to provide stereoselectivity. A fractional factorial design involving eleven separate experiments was used to generate the required data. The methods were optimized for percentage of organic in the mobile phase, concentration of cyclodextrin in the mobile phase, mobile phase buffer pH and stationary phase. Analysis of the data showed which of the factors needs to be critically controlled for robustness of the methods.

INTRODUCTION

Lometrexol sodium and LY231514 are structurally similar compounds of pharmaceutical interest as anti-tumor agents. Lometrexol (Figure1), with two chiral centers has the potential of generating four stereoisomers. Because the stereochemistry at one of the chiral centers is determined early in the synthetic process using high-purity reagents, the potential for formation of two of the

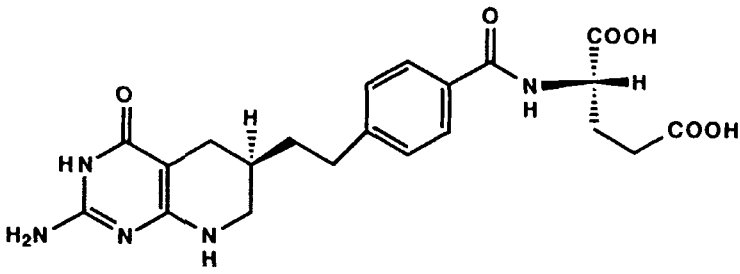

Figure 1. Structure of Lometrexol.

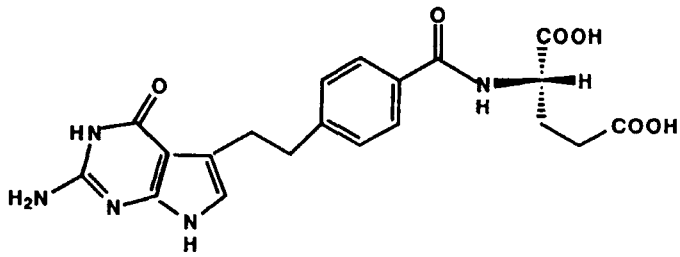

Figure 2. Structure of LY231514.

stereoisomers is negligible. The other pair of isomers, lometrexol and a diastereomeric impurity, must be analyzed by a suitable method. Although in theory these diastereomers should be separable by reversed phase HPLC in an achiral environment, repeated attempts to effect a separation using a variety of columns and conditions were unsuccessful. Thus, a chiral environment using beta-cyclodextrin as a mobile phase additive to a reversed phase HPLC system was invoked to effect a separation of the isomers.

In contrast to lometrexol, LY231514 (Figure 2) has a single chiral center. A chiral assay is required to assess the level of the undesired enantiomer. A similar assay to the one employed to separate lometrexol from its diastereomer using beta-cyclodextrin as a mobile phase additive to a reversed phase system was found to achieve a separation of LY231514 enantiomers.

Cyclodextrins have been utilized in liquid chromatographic chiral separations both as mobile phase additives as well as in bonded phases.[1-4] The approach of adding cyclodextrins to the mobile phase offers the advantages of lower cost and less concern about column ruggedness. In addition, the mobile phase additive approach offers more ways to optimize retention and selectivity.

Optimization of a method involving isomeric separation has the aim of achieving maximum resolution with minimum assay time. Robustness testing must be done to determine which operating conditions will significantly effect resolution and retention time in order to determine the degree of control required. Parameters may include: buffer pH and ionic strength, column type and temperature, type and amount of organic modifier, and flow rate. These methods were initially optimized to some extent by varying individual parameters independently, a traditional approach.[2,5,6] This practice is relatively laborious and makes it difficult to fully characterize interdependence of the parameters.

Another approach which can be used for method optimization and robustness testing is an experimental design.[7-8] Experimental designs are useful at all stages of method development and optimization. For compounds in early clinical studies a screening design with minimal experiments can be used to determine the important method parameters. For methodology included in a product registration, a full factorial design can determine the impact of method parameters and all interactions between those parameters. Described in this work is a fractional factorial design which allows for the determination of some interactions.

EXPERIMENTAL

Chromatographic experiments were carried out using an Spectra-Physics P1500 HPLC pump equipped with a Kratos 757 UV detector, Micromeritics 728 autosampler and Reodyne 7010 injection valve. Fixed variables included flow rate (1.5 mL/min.), column temperature (30°C), detector wavelength (270 nm) and injection volume (20 μL). HPLC columns were 25 cm Phenomenex IB-SIL ODS and IB-SIL C1 with 5 micron particles.

HPLC grade acetonitrile (ACN) and water were used. Beta-cyclodextrin (ß-CD) was purchased from Ensuiko Sugar Refining Company, Ltd. Lometrexol, LY231514 and their associated isomers were synthesized in Lilly Research Laboratories. All experiments were conducted with a sample prepared at 50/50 ratio of desired/undesired isomer. JMP® statistical software was used to generate the experimental design.

RESULTS AND DISCUSSION

Initial Method Development and Optimization

Initial optimization of the isomeric purity assay for lometrexol involved testing one variable at a time. For each variable tested, resolution between lometrexol and the major diastereomer was measured and retention time noted. Parameters tested included flow rate, column temperature, ß-CD concentration, ACN/buffer ratio, buffer type and pH. The final mobile phase consisted of a 97% buffer (1% triethylamine-phosphate at a pH of 7.0) and 3% ACN with 8 g/L ß-CD. The flow rate was set at 1.5 mL/min. and column temperature at 30°C. The column selected was a 25 cm Phenomenex IB-SIL ODS with 5 micron particles. Assay time is 30 minutes.

The assay for LY231514 was adopted from the method conditions for lometrexol. Slight modifications were made to the mobile phase and flow rate to adjust retention time. The mobile phase was 96.5% buffer (1% triethylamine-phosphate at a pH of 7.0) and 3.5% ACN with 6 g/L ß-CD. The flow rate was set at 2.0 mL/min. and column temperature at 30°C. The column was a 25 cm Phenomenex IB-SIL ODS with 5 micron particles. Assay time is 42 minutes.

As these compounds progressed through clinical studies towards efficacy testing the sample load increased. In addition, the possibility of transferring methodology to other laboratories developed. The methods were reviewed and the decision made to use an experimental design to optimize assay conditions with the goal of shortening assay time and decreasing the ß-CD concentration. The high levels of ß-CD had a detrimental effect on the HPLC column and pump.

Factorial Design

A full factorial design is used to determine the main effects and all interactions between the factors selected. The number of trials necessary is 2^k, where k is the number of factors. For lometrexol and LY231514 methods the number of factors can include ß-CD concentration, stationary phase, buffer pH, buffer/organic modifier ratio, flow rate, and column temperature. Evaluating all of these parameters with a full factorial design would involve $2^6 = 64$ trials. This represents a significant amount of experimental time.

Table 1

Experimental Design Factors and Values

Factors	Parameter	+1	-1
X1	% Acetonitrile	2	10
X2	g/L β-CD	2	8
X3	Buffer pH	3	7
X4	Stationary Phase	C1	C18

Table 2

Fractional Factorial Design

Trial	Factor			
	X1	X2	X3	X4
1	-1	-1	-1	-1
2	0	0	0	0
3	-1	-1	+1	+1
4	-1	+1	+1	-1
5	+1	+1	-1	-1
6	0	0	0	0
7	-1	+1	-1	+1
8	+1	+1	+1	+1
9	+1	-1	+1	-1
10	0	0	0	0
11	+1	-1	-1	+1

In order to minimize experimental time, factors were carefully evaluated in light of what had been learned during the lometrexol method development. For example, limited testing of the impact of column temperature had found no significant change to retention time or resolution in a range of 30°C - 50°C.

Table 3

Experimental Results

Trial	Lometrexol		LY23154	
	Rtention Time (min.)	**Resolution**	**Retention Time (min.)**	**Resolution**
1	80	3.8	250	0
2	18	2.2	37	1.8
3	116	3.8	196	1.8
4	9	1.9	21	1.6
5	4	1.7	11	0
6	20	3.2	38	1.8
7	45	4.4	170	0
8	3	0.8	4	0.9
9	4	0.6	5	0
10	19	3.3	15	1.7
11	11	2.2	24	0

Therefore, column temperature was not considered a critical factor. Similar data existed regarding flow rate. Three key factors were selected, ß-CD concentration, buffer/ACN ratio, and buffer pH. A fourth factor, stationary phase was also selected.

The four factors in a full factorial would require 16 trials. This investment in experimental time is not extensive and would be more than appropriate for optimization of methodology intended for regulatory registrations. However, for these compounds, the goal was to improve existing methodology with a minimum amount of time. Therefore a fractional factorial design was selected. Fractional factorials measure main effects and some interactions with the number of trials is 2^{k-p} where p is an arbitrary number less than k. For these experiments p = 1, and the number of trials was $2^{4-1} = 8$.

Selection of maximum (+1) and minimum (-1) values for the factors is critical for generation of useful data. Practical considerations such as ß-CD solubility and stationary phase pH stability place limitations on value selection. However key information can be found from method development experiments. Factors selected and their +1 and -1 values are shown in Table 1.

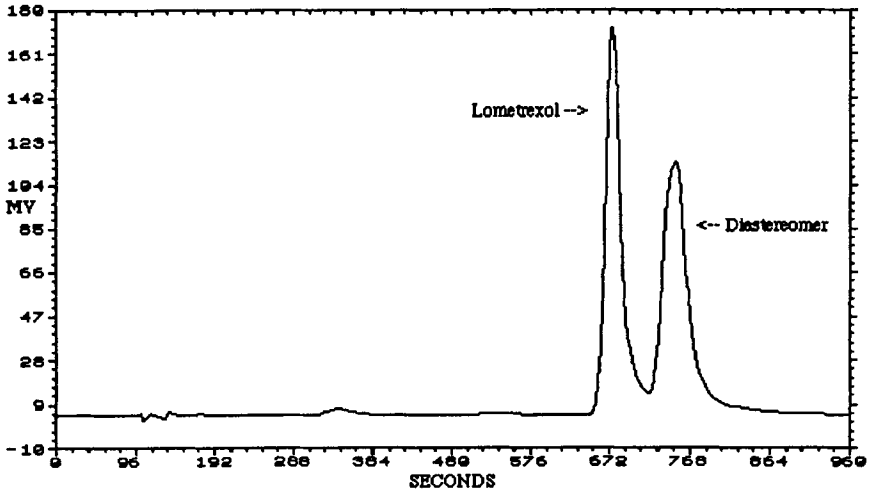

Figure 3. Chromatogram of Lometrexol separation.

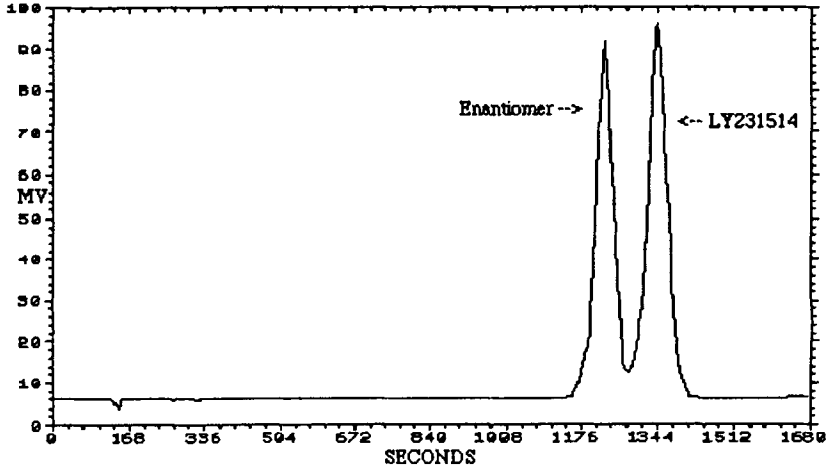

Figure 4. Chromatogram of LY231514 separation.

The fractional factorial design is shown in Table 2. In addition to the eight trials three center points were added. These serve as a control of system performance.

The same HPLC equipment, analyst and sample was used for all experiments. Because the design involved constant changing of column and mobile phase, a process for column and instrument rinsing was defined and used throughout the experiment.

The results of the trials for both compounds are shown in Table 3. Statistical analysis of the data using JMP software shows that the factor with the greatest significance on retention time is, not unexpectedly, the ACN concentration. For resolution, the most significant factor is buffer pH.

Additionally, the data showed that a decrease in ß-CD concentration combined with an increase in %ACN would maintain or improve the resolution obtained with the current method conditions. Column type had no statistical effect on either resolution or retention time.

CONCLUSIONS

Use of a fractional factorial design with 11 experiments identified buffer pH as a significant factor in controlling resolution for both compounds. In addition, slight modifications in %ACN and ß-CD concentration will shorten assay time and maintain or improve resolution. This points the way to future method development.

For lometrexol, the data shows that changing the buffer pH from 7 to 3, decreasing the ß-CD concentration from 8 g/L to 2 g/L and increasing the %ACN from 3% to 10% will shorten assay time by 14 minutes and improve resolution (Figure 3). For LY231514, a decrease in %ACN (from 3.5% to 2%) combined with an increase in ß-CD (from 6 g/L to 8 g/L) will shorten assay time by 14 minutes and increase resolution (Figure 4).

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