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Journal of Chromatography A, 844 (1999) 171–179

JOURNAL OF CHROMATOGRAPHY A

Optimization and validation of chiral high-performance liquid chromatographic method for analysis of a fibrinogen (gpIIb/IIIa) receptor antagonist

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Received 3 August 1998; received in revised form 26 January 1999; accepted 27 January 1999

Abstract

A chiral HPLC separation on an α_1-acid glycoprotein (AGP) column was developed for the separation of roxifiban, a fibrinogen receptor antagonist, from its related chiral impurities. The proposed method was optimized for mobile phase buffer concentration, organic modifier, and temperature using experimental design. The method was then validated for use in release testing of the roxifiban drug substance.

Keywords: Chiral stationary phases, LC; Roxifiban; Fibrinogen receptor antagonist

1. Introduction

The isoxazolines are a relatively recent group of non-peptide platelet gpIIb/IIIa antagonists being developed as potential antithrombotic agents [1]. The compounds can be characterized by the presence of one or more chiral centers and their preparation in optically pure form poses synthetic challenges. Of these compounds, roxifiban (DMP 754) has emerged as a stable and pharmaceutically suitable drug substance for development as a drug candidate [2,3]. Roxifiban (Fig. 1) is the acetate salt of a carboxymethyl ester prodrug with two chiral centers and is being developed as a single stereoisomer prodrug. Since achiral reversed-phase and normal-phase HPLC methods using columns with C_{18}, C_8, cyano, and phenyl stationary phases could not separate the diastereoisomers from the roxifiban, a chiral method had to be developed for analysis of the three potential chiral impurities in the drug substance.

After some investigation a promising chiral HPLC method with a Chiral AGP column was found that could separate two of the three chiral impurities in the drug substance. The Chiral AGP column has an α_1-acid glycoprotein stationary phase bound to a silica support [4,5] and cross linked to cover the silica surface and a number of chiral separations have been reported in the literature. Method development of complex samples on the Chiral AGP column can be complicated since selectivity is affected by a variety of factors such as mobile phase pH, organic solvent modifier, temperature, etc. [6,7], and experimental design can potentially minimize the necessary method development work. Experimental design techniques have been previously used with the Chiral AGP column [8,9] and other chiral

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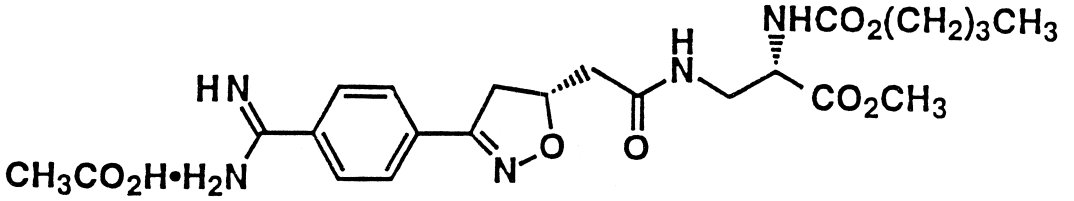
0021-9673/99/$ – see front matter
PII: S0021-9673(99)00172-7



Roxifiban

Fig. 1. Structure of roxifiban.

methods [10,11]. However, none of these techniques were used to optimize methods simultaneously for speed and separation of chiral impurities in drug substance samples. In view of the number of mobile phase variables that could be investigated in the roxifiban chiral separation, a screening experiment was needed to help identify three suitable variables that could then be used in further optimization studies with response surface methodology.

The objective of this study was to develop an optimized chiral separation method using experimental design techniques that would separate the four stereoisomers of DMP 754 in the minimum time with sufficient sensitivity to measure any low levels of chiral impurities present in the drug substance.

2. Experimental

Roxifiban (structure in Fig. 1) has a basic benzamidine group which has a pKa of 11 and exhibits a strong native fluorescence in aqueous solvent with excitation and emission wavelengths of 282 and 375 nm respectively. This prodrug is being developed as a single stereoisomer in the RS configuration. The RR and SS diastereoisomers and the SR enantiomer are potential synthetic impurities which need to be analyzed in the drug substance. Standards of these stereoisomers were all prepared by the Chemical Process Research and Development section of the DuPont Pharmaceuticals Company (Wilmington, DE).

The chromatographic equipment used in this work consisted of a Hewlett-Packard Model 1050 HPLC system equipped with an autosampler, column oven and a Hewlett-Packard Model 1046A fluorescence detector with a xenon lamp and monochromators to select excitation and emission wavelengths. The fluorescence detector was set up with a 345 nm excitation cut off filter to remove stray light and a photomultiplier gain setting of 15 was used for all measurements. The detector output was interfaced to a Vax based data collection system (Multichrom version 1.8-3, Fisons U.K.) from which the chromatograms were generated.

A Chromtech Chiral AGP column, 100×4 mm, with 5 micron packing (obtained from Advanced Separations Technology, Whippany, NJ) was used for the chiral separation. Reagent grades of isopropanol (IPA) and glacial acetic acid, and HPLC grades of methanol (MeOH), acetonitrile (ACN), and tetrahydrofuran (THF) were obtained from EM Science (Gibbstown, NJ). The mobile phases and sample solvents were prepared using reagent grade sodium phosphate monobasic, monohydrate, (EM Science, Gibbstown, NJ) which was titrated to the appropriate pH with 1N NaOH (EM Science, Gibbstown, NJ) and the organic solvent modifier was then added to obtain the appropriate composition.

The screening experiments and experimental designs were both constructed and the data analyzed using statistical software (JMP version 3.2, SAS, Carey NC)

3. Results and discussion

3.1. Chiral separation

The Chiral AGP column was selected for further investigation after preliminary attempts to resolve the diastereoisomers of roxifiban on other chiral columns

with cyclodextrin, macrocyclic antibiotic, and derivatized cellulose and amylose stationary phases proved to be of limited success. Significant problems associated with earlier attempts to resolve these stereoisomers included excessive tailing of the peaks associated with the basicity of the benzamidine group (pKa 11). Initial separations on the AGP column (Fig. 2) indicated the feasibility of resolving at least two of the three chiral impurities in the drug substance. Although the Chiral AGP column has been widely used for many years to separate a variety of chiral compounds, it has often not been the

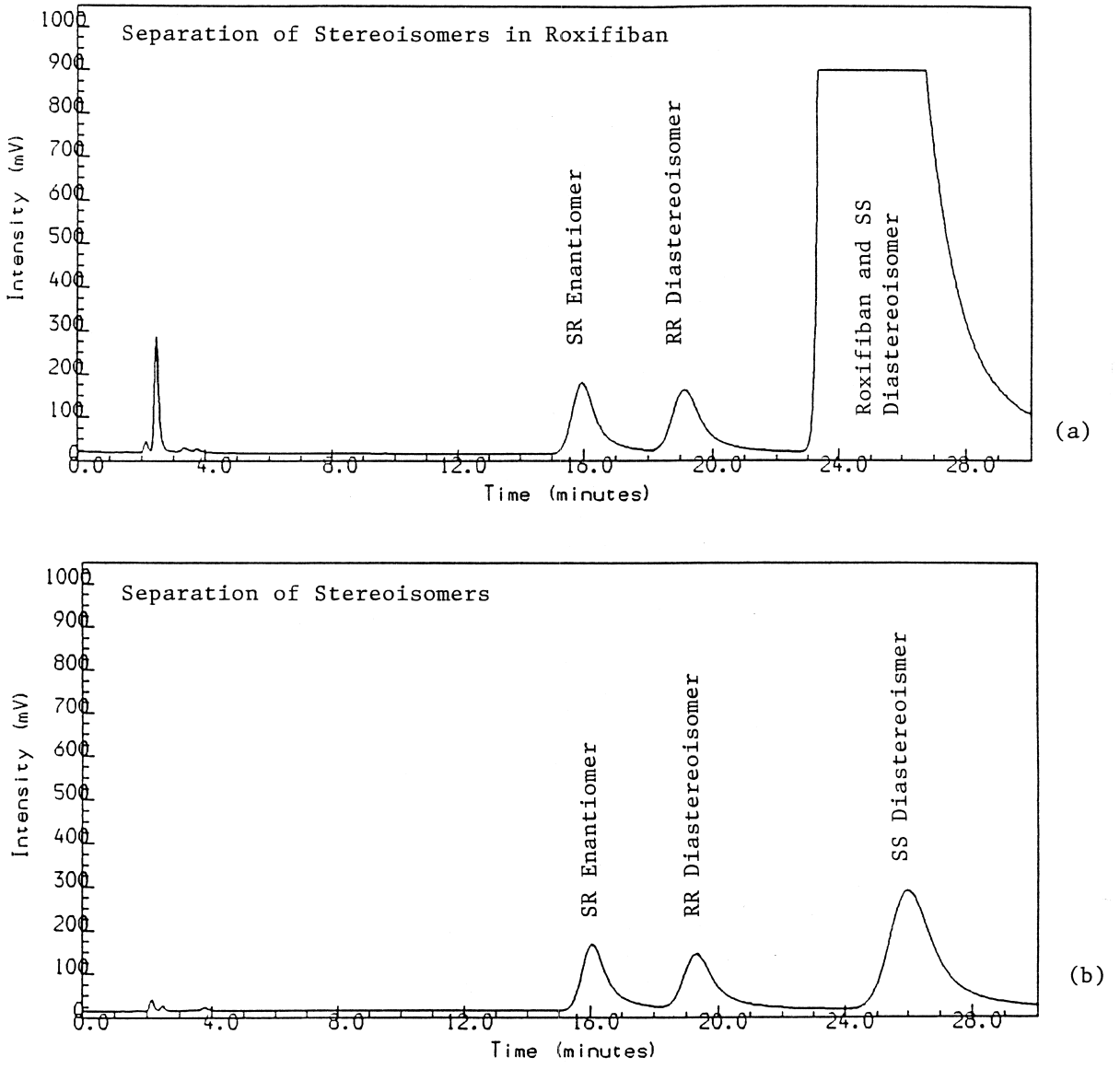


Fig. 2. Chromatogram a, separation of stereoisomers in roxifiban drug substance; Chromatogram b, separation of stereoisomer standards: Chromatographic conditions: Chiral AGP column 10×0.4 cm; mobile phase of 97% 20 m*M* sodium phosphate pH 6.0 and 3% ACN; flow rate of 0.2 ml/min; column temp of 25°C; fluorescence detection with 282 nm excitation and 375 nm emission; sample of 5 ng SR and RR, 10 ng SS, 0.5 μg roxifiban.

Table 1
Effect of sample amount on DMP 754 peak

Sample size (μg)	Retention time (min)	Peak width (half height) (s)
0.05	26.50	224
0.10	26.22	224
0.20	25.64	224
0.25	25.58	224
0.50	24.72	248
1.00	23.62	288
2.00	22.48	352
4.00	20.24	384

column of choice for analysis of low levels of chiral impurities in complex samples. The Chiral AGP column usually gives relatively broad sample peaks (less than 3000 theoretical plates) and has a relatively low sample capacity [12,13]. The low sample capacity is a problem when analyzing low levels (0.1%) of chiral impurities in large drug substance samples since only limited (microgram) amounts of sample can be injected before significant peak broadening occurs because of column overloading effects. This makes resolution and detection of impurity peaks difficult. Fortunately, the roxifiban molecule has a strong native fluorescence so that detection and quantitation of even subnanogram quantities of the stereoisomer impurities is possible with a sensitive fluorescence detector. The fluorescence detection is also specific since the drug substance contains few other impurities which fluoresce and could interfere with the chiral separation.

The chromatograms in Fig. 2 show the initial separation of the the chiral impurities and a 0.5 microgram sample of drug substance spiked with these impurities at the 1.0% level. The peak broadening effect from sample overload of the drug substance (RS stereoisomer) is significant and makes separation from the SS stereoisomer difficult. The effect of sample overload on retention and peak width can be seen in Table 1 where increasingly larger amounts of roxifiban were chromatographed. It was decided to do all further development with samples of no more than 0.5 micrograms of roxifiban drug substance in order to minimize column overload effects while maintaining sufficient sensitivity to detect and quantitate the chiral impurities.

3.2. Choice of mobile phase variables using screening experiments.

Although the separation in Fig. 2 was promising, it was necessary to further develop the separation so that the SS stereoisomer could be resolved and analyzed in drug substance samples. In recognition of the number of variables influencing the selectivity of the Chiral AGP column a screening experiment was conducted to reduce the number of variables of interest. Since the factors of mobile phase pH and mobile phase organic modifiers (MeOH, IPA, ACN, and THF) strongly affect column selectivity [6,7], a five factor, two level screening experiment [14,15] was employed to identify which of these variables should be used in further optimization experiments.

As illustrated in Table 2 the five factors of pH,

Table 2
Design of five factor, two level screening study

	pH	%IPA	%MeOH	%ACN	%THF			
Low	4.5	0	0	0	0			
High	7.0	3	10	3	2			
Experiment	Pattern	pH	%IPA	%MeOH	%ACN	%THF	R_1	R_2	R_3
1	− − + + −	4.5	0	10	3	0	0.2	0.2	0.5
2	+ + − − −	7	3	0	0	0	4.7	2.3	2.0
3	− + − + −	4.5	3	0	3	0	0.7	0.5	1.0
4	− − − − +	4.5	0	0	0	2	0.2	0.5	0.5
5	+ − − + +	7	0	0	3	2	1.4	0.8	1.2
6	+ + + + +	7	3	10	3	2	0.7	0.3	1.0
7	+ − + − −	7	0	10	0	o	5.0	1.3	4.4
8	− + + − +	4.5	3	10	0	0	0.8	0.3	1.0

%MeOH, %IPA, %THF, and %ACN were each assigned a high and low level. The low level of pH was selected to be 4.5 (close to lower limit of column and sample stability) and the high level was chosen to be 7.0 which is the upper limit of both column and sample stability. The low level of each modifier was chosen to be zero; the high levels were selected so as to insure sufficient retention of the solutes on the column for meaningful measurements of resolution. These high level modifier values were estimated knowing the retention of roxifiban shown in Fig. 2 and also from previous experience in our labs on the relative effect of the four organic solvent modifiers on the retentivity of the Chiral AGP column.

Samples containing standards of all four stereoisomers were injected in each experiment listed in Table 2. Individual stereoisomers were also injected when necessary to help determine retention and to measure or estimate resolution factors. The resolution factors [16] between the SR and RR stereoisomers, R_1, between the RR and SS stereoisomer, R_2 and between the SS and roxifiban peaks, R_3, were measured at peak half height for the 8 experiments and results shown in Table 2. These resolution factors were then used with the JMP software to generate the profile plots displayed in Fig. 3. Each profile plot showed the effect of the changing one factor (pH or % modifier) on the predicted resolution while holding the other factor settings constant.

The profile plots in Fig. 3 indicate that best resolution of the stereoisomers is obtained at pH 7. The results also show that the organic modifiers acetonitrile and tetrahydrofuran tend to diminish resolution between all peak pairs and were thus excluded from further consideration. Increase in

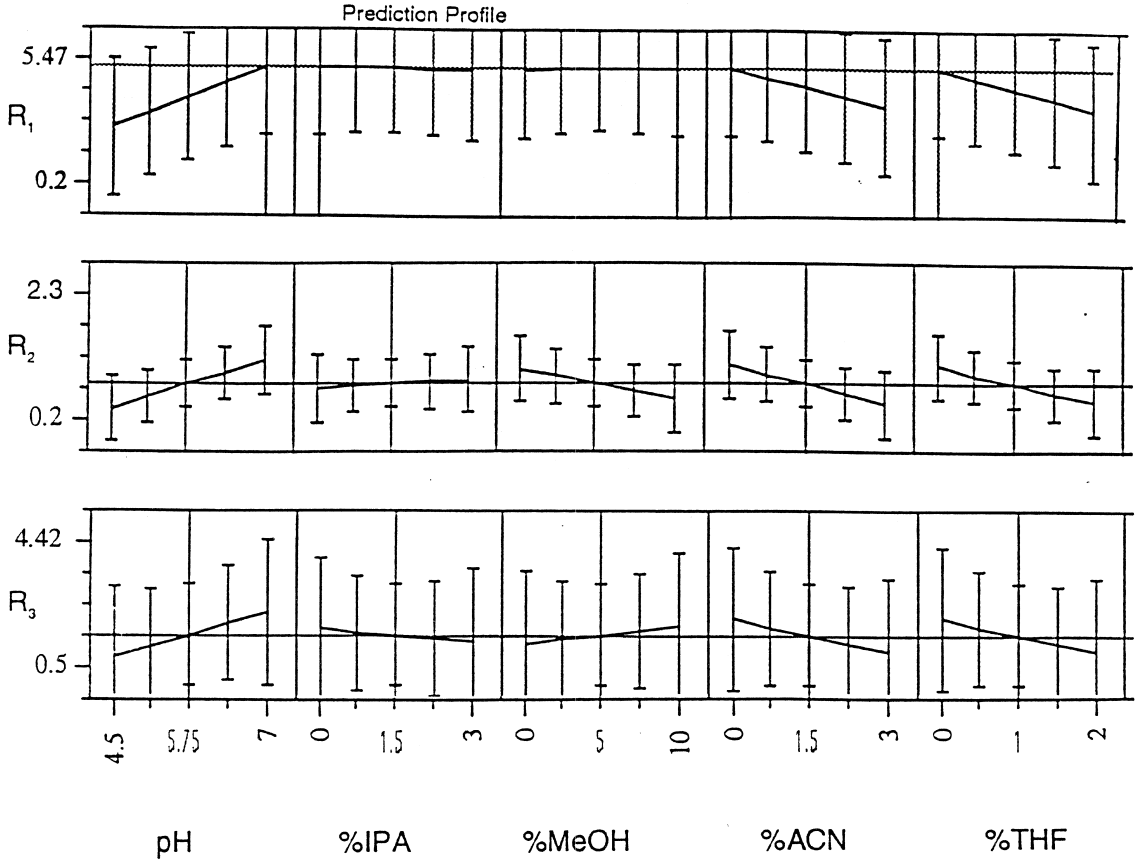


Fig. 3. Resolution factor versus pH and % modifier.

methanol concentration exhibited opposing effects on the resolution between peak pairs and was also excluded. Isopropanol was chosen for further optimization studies since increasing its concentration reduces retention with minimal effect on resolution.

3.3. Optimization by experimental design

Following preliminary screening experiments, the three variables of %IPA, sodium phosphate buffer concentration, and temperature were selected for method optimization using a response surface design since these variables were known to affect retention and selectivity of the separation. A pH of 7 was used for all experiments since the screening experiments clearly showed that it gave the best resolution of all stereoisomers. A three factor, two level fractional factorial design with center point [17,18] was then chosen for the experimental work. The high, low and center point levels of the three factors are listed in Table 3 with the first eight experiments representing the corner points of the fractional factorial. The center point was repeated three times to establish the precision of the measurements and the order of experiments was randomized. Samples containing 0.5 micrograms of roxifiban and 5 nanograms of each of the chiral impurities were injected in each experiment to determine separation. A sample containing only the RR and SS stereoisomers was also injected in each experiment to verify retention and peak identity.

For these separations a composite criterion was developed to take into account the need for adequate resolution between peak pairs without unduly prolonging the analysis time. As the SR enantiomer was well resolved from the other stereoisomers it was not considered in developing a criterion. Additionally since the resolution factor between the small SS and comparatively large roxifiban peak was difficult to measure and calculate, the valleys between the peaks were measured (the method of calculation is shown in Fig. 5) and used in the fractional factorial design.

Table 3
Design of three factor, two level fractional factorial study

Pattern	Block	% IPA (v/v)	Buffer (m*M*)	Temperature (°C)	V_2V_3/t_r
− − −	1	3	10	22	0.0187
− − +	1	3	10	30	0.00863
− + −	1	3	50	22	0
− + +	1	3	50	30	0
+ − −	1	7	10	22	0.0148
+ − +	1	7	10	30	0.0211
+ + −	1	7	50	22	0.0184
+ + +	1	7	50	30	0.0155
0	1	5	30	25	0.0356
0	1	5	30	25	0.0353
0	1	5	30	25	0.0352

The best criterion developed to optimize for both speed and separation was defined as the product of the valleys between adjacent peaks divided by the analysis time, V_2V_3/t_r where t_r is the retention time of roxifiban. Larger values of V_2V_3/t_r represent better combinations of speed and separation. The V_2V_3/t_r response parameter was calculated for the 11 experiments and listed in Table 3 and used in the calculations of the fractional factorial study.

The response surfaces obtained on fitting a quadratic relationship are illustrated as the contour plots shown in Fig. 4. The response surfaces obtained suggest that temperature has minimal effect on selectivity but predict an optimum combination of separation and speed at 5% IPA and 10 m*M* buffer concentration. It was then experimentally confirmed that this mobile phase was able to resolve the SR, RR and SS stereoisomers from roxifiban (see Fig. 5) with an optimum of resolution and speed. A typical drug substance sample with chiral impurity is also shown in Fig. 5.

4. Validation

The optimized separation (Fig. 5) was then validated for precision, limits of detection and quantitation, accuracy, linearity and robustness. The precision of peak measurement was determined by measuring peak height and areas of six injections of a sample of the containing 2.5 ng each of the RR, SS, and SR stereoisomers. The RSD of both peak height and peak area measurements were determined to be less than 2.0% for each standard. The limits of detection (3 times signal-to-noise) and quantitation (10 times signal to noise) was measured to be 0.2 and 0.5 nanograms respectively.

Accuracy and repeatability of the method was

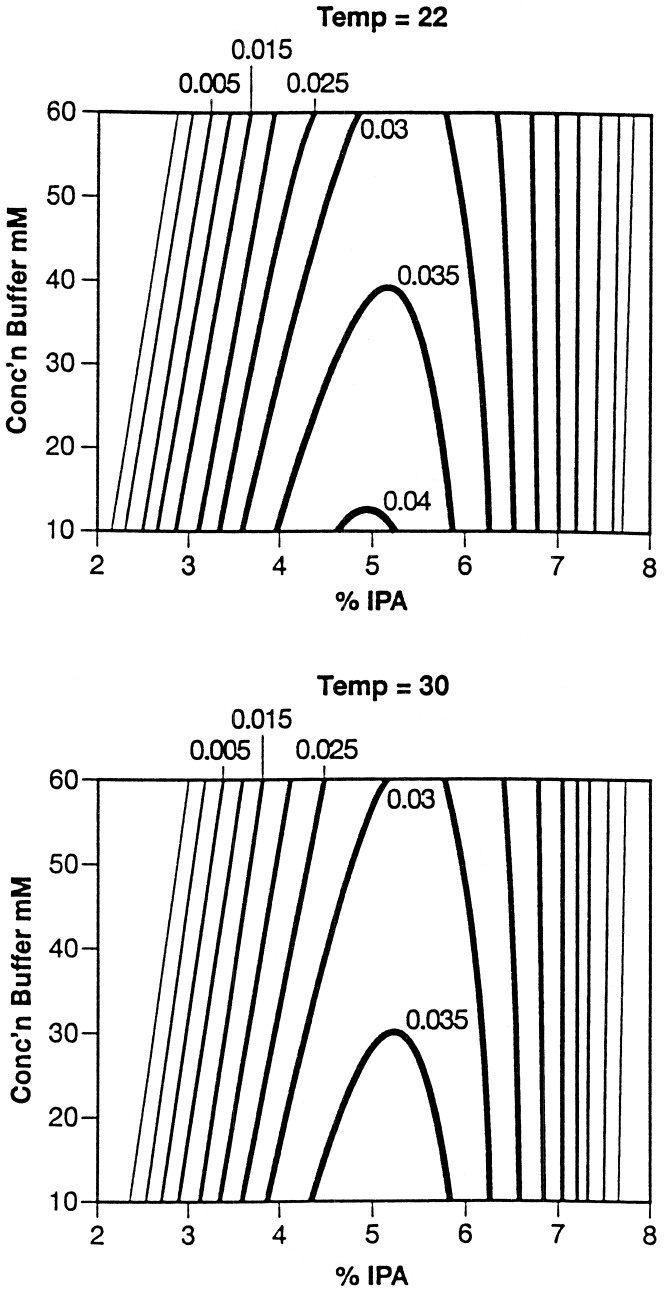


Fig. 4. Contour plots of roxifiban separation. Response surface of V_2V_3/t_r plotted against % IPA and m*M* buffer.

measured with a recovery study. Small amounts (0.5, 2.0, 2.5, and 3.0 nanograms) of each of the RR, SS, and SR stereoisomers were spiked into separate 0.5 microgram samples of roxifiban drug substance. Peak heights of these spiked stereoisomers were measured and compared to peak heights of stereoisomer standards in samples containing 50 nanograms of roxifiban in order to calculate recoveries. Small amounts of the roxifiban drug substance were added to the stereoisomer standard samples in order

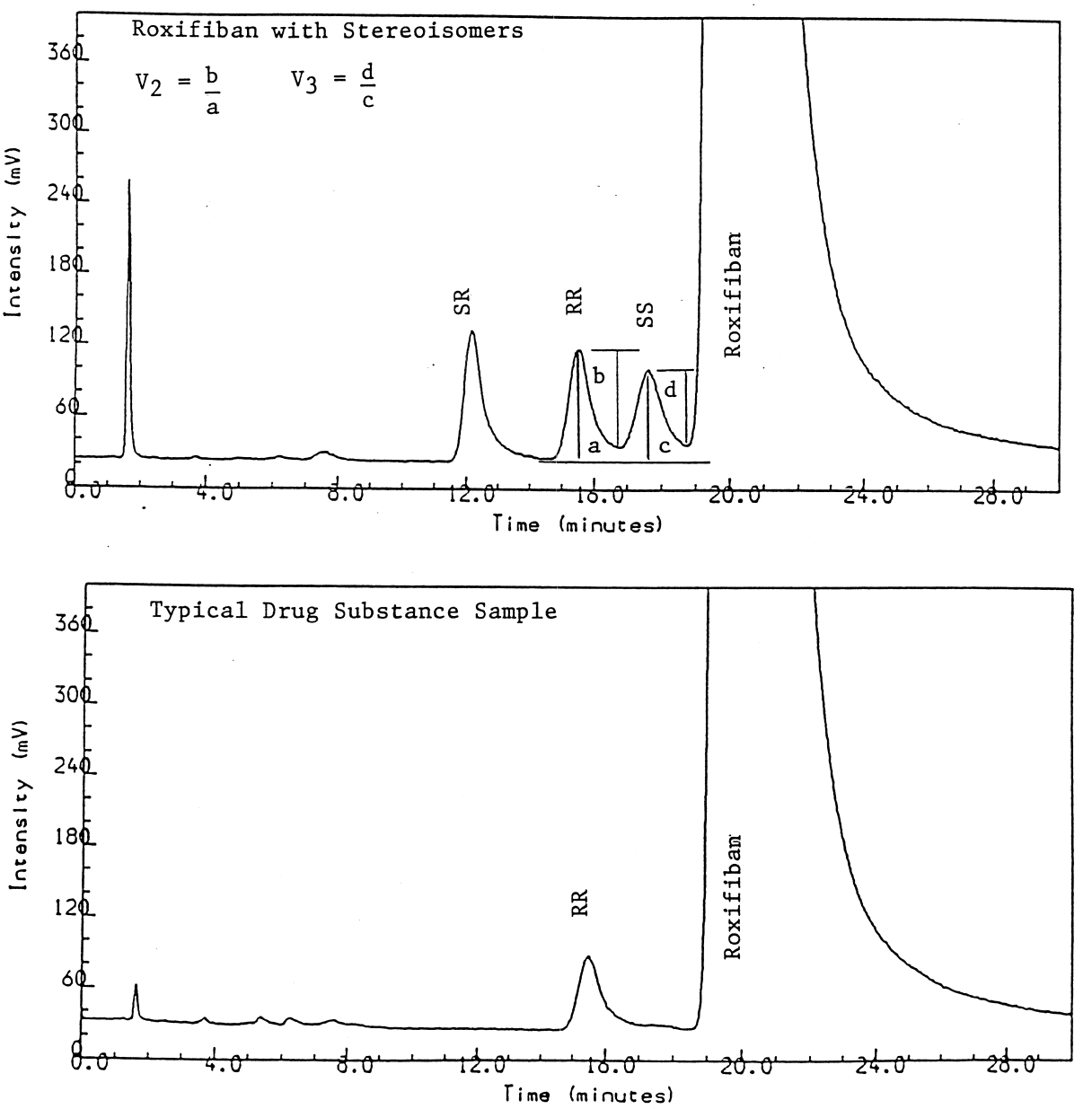


Fig. 5. Optimized separation of roxifiban drug substance and stereoisomers chromatographic conditions: Chiral AGP column; mobile phase of 95% 10 m*M* sodium phosphate pH 7.0 and 5% IPA; 0.9 ml/min; 25°C; fluorescence detection with 282 nm excitation and 375 nm emission; sample of 2.5 ng each SR. RR, SS and 0.5 μg roxifiban in A, 0.5 μg roxifiban in B. Measurement of valley between diastereoisomer peaks is defined as the depth of valley divided by height of peak; these ratios are used in the composite criterion for optimizing the separation.

to prevent irreversible adsorption of small amounts of the standards on the Chiral AGP column packing. Average recoveries for triplicate samples were then calculated to be 97.6% for the RR, 101.7% for the SS, and 101.8% for the SR stereoisomers; the %RSD was 2.5%, 4.2% and 6.3% respectively The linearity

of the peak height calibration curves were measured using standards of 0.5, 2.0, 2.5, 3.0, and 5.0 nanograms; the calibration curves were all linear through zero with correlation coefficients of 0.9999, 0.9999, and 0.9997 for the SR, RR and SS stereoisomers respectively.

The separation shown in Fig. 5 was replicated on three different columns from different batches of column packing to demonstrate the robustness of the method.

5. Conclusions

A method for the chiral HPLC separation and analysis of the isoxazoline roxifiban from its three stereoisomers has been developed using experimental design. We have demonstrated that a combination of considerations such as limitation on sample loading of the Chiral AGP column, column selectivity, and the need to obtain analytical sensitivity can be coupled with experimental design techniques to optimize the separation conditions for both speed and selectivity of the chiral HPLC method. A five factor two level screening experiment was first used to identify the important chromatographic variables in 8 experiments. The separation was further optimized for both speed and resolution with a three factor two level fractional factorial response surface design in 11 more experiments. The optimized method was then validated for use in the analysis of chiral impurities in the roxifiban drug substance.

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