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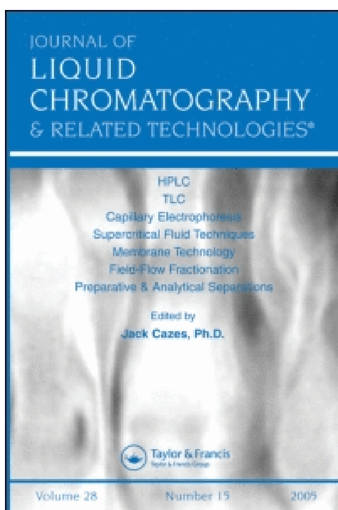

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MODELING THE SELECTION OF FRACTIONS DURING PREPARATIVE HPLC OF A SEMISYNTHETIC PNEUMOCANDIN

Paul D. Oram[a]; Kevin Seibert[a]; William Leonard[a]; Angelos Dovletoglou[a]
[a] Merck Research Laboratories, Merck & Co., Inc., Rahway, NJ, U.S.A.

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MODELING THE SELECTION OF FRACTIONS DURING PREPARATIVE HPLC OF A SEMISYNTHETIC PNEUMOCANDIN

Paul D. Oram,* Kevin Seibert, William Leonard, and Angelos Dovletoglou

Merck Research Laboratories, Merck & Co., Inc.,
P.O. Box 2000, Rahway, NJ 07065-0900, USA

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ABSTRACT

Analytical high-performance liquid chromatography (HPLC) data collected during developmental scale-up studies were used to design an empirical model that was applied to the selection of HPLC fractions obtained during preparative scale reverse-phase HPLC of a semisynthetic lipopeptide antifungal. These data resulted in the ability to collect fractions containing the target compound at acceptable purity and with minimal yield loss, based on the ultraviolet data generated by the preparative column detector. The methodology used was successfully integrated from a developmental scale to a manufacturing scale.

INTRODUCTION

The antifungal drug substance candidate caspofungin acetate is synthesized from a compound that is isolated from a fermentation broth (1–3). Reverse-phase

*Corresponding author.

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large-scale high-performance liquid chromatography (LSHPLC) is used for purification of caspofungin and one of the intermediate compounds during the organic synthesis. A mathematical model that predicts the elution behavior of the batch components would be useful for selection of rich cuts. Such modeling has been successfully applied to binary mixtures (4). In the case of a fermentation-derived compound, however, there are a large number of impurities, and the structures of many of these remain unknown. Furthermore, a number of the impurities, whose identities are known, are difficult to resolve from the main compound on the preparative column. One technique for monitoring unresolved peaks in a preparative column eluent is to use on-line analytical HPLC (5). However, the shortest possible analytical HPLC run time available for this process (12 min) is too slow compared to the rate at which the levels of impurities change as they elute from the preparative column. Therefore, during scale-up runs for the production of caspofungin, fractions from the LSHPLC were collected and held while analyses were performed in the laboratory. The results of these analyses were used to determine which cuts could be combined for further processing.

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In addition to the HPLC run time, the time required for sampling, sample transfer, and data reduction all add to the hold time for the fractions. This delay is undesirable in any production process, but is even less so for this compound, since there is the potential for degradation to occur in solution. The goal for commercial production is to collect only one center cut per injection and to continue processing cuts with minimum delay. Data collected during the scale-up runs were utilized to establish certain criteria that can be used for automated rich-cut selection in the preparative chromatography of this compound. This approach has been described recently for the selection of high-boiling petroleum fractions and their processing products (6), and it is shown in this paper that in general the methodology can be adapted and applied to other applications, such as the purification steps of a complicated pharmaceutical process.

EXPERIMENTAL

The commercial production process involves synthetic modification of a fermentation broth-derived starting material. Preparative LSHPLC was used for purification during two steps of this synthesis (step A and step B). Step A is a reduction process that results in the intermediate compound IA, shown in Figure 1. Step B is a displacement reaction that results in the final compound, IB (caspofungin), also shown in Figure 1. The preparative chromatography in both steps was performed on a 30-cm diameter Prochrom HPLC column (Prochrom, Champigneulles, France). A Biotage KP-3000 pumping skid (Biotage Inc., Charlottesville, VA, USA) with a PC interface, running a custom FixDMacs software package (Intellution, Norwood, MA, USA), was used to capture all ultravio-

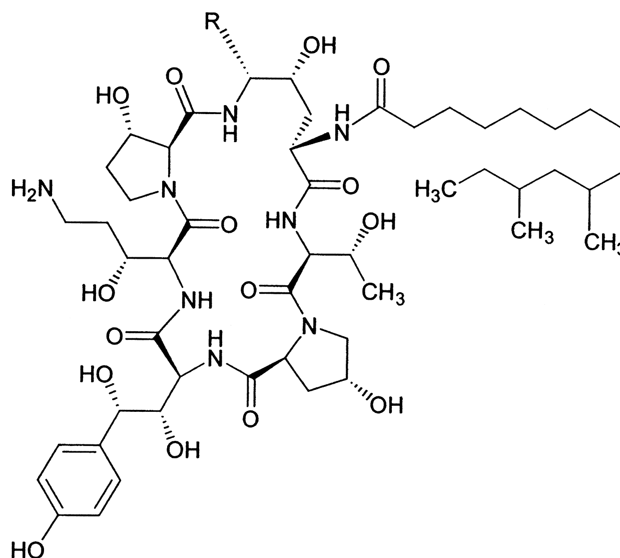

Figure 1. Structure of step A reduction intermediate [IA] ($R = \frac{1}{n}SC_6H_5$) and of the final product from the step B displacement reaction, caspofungin [IB] ($R = -NHCH_2CH_2NH_2$).

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let (UV) absorbance, flow, and pressure data. The 30-cm diameter column was packed to a depth of 29 cm with Kromasil, 16 μm, 100 Å, C^{18} modified silica (Eka Chemicals, Bohus, Sweden).

Tracer tests were used for measuring the theoretical plate count of newly packed columns and for verification of packing performance between injections. Tracer tests were performed by elution of a 1% solution of propyl 4-hydroxybenzoate (Fisher Scientific, Houston TX, USA), with saturated uracil (Fisher Scientific) as an unretained species in the eluent. The eluent used for all tracer tests was 60% (v/v) acetonitrile/water (acetonitrile from BP Chemicals, Cleveland, OH, USA). Overall plate count, N, was determined using the half-height method [Eq. (1)],

$$N = 5.54 \left(\frac{t_R}{w_{1/2}}\right)^2 \qquad (1)$$

where t_R is the time for the retained species to exit the column from the time of injection, and $w_{1/2}$ is the width of the peak, in units of time, at half-maximum height. The conditions for elution during step A and step B are shown in Tables 1 and 2, respectively. Fractions were collected at given time points using the UV absorbance spectra as a guide. The timing of fraction collection was varied, depending on the level of detail needed to establish a profile of the impurities as they elute from the column. For example, in step A, 5-min intervals in the front

Table 1. Range of Preparative Column Operating Parameters During Step A LSHPLC Injections[a]

Chromatographic Parameter	Operating Range
Column condition (10% ACN)	5 BV
Condition flow rate	8.7–18.9 BV/h (11.7)
Feed volume	7.7–18.1 BV (9.7)
Feed flow rate	4.1–6.4 BV/h (5.8)
Eluent 1 (29% ACN with acid)	9.7–12.1 BV
Eluent 1 flow rate	6.1–10.8 BV/h (9.9)
Eluent 2 (29% ACN)	25–30 BV (to end of IA peak)
Eluent 2 flow rate	11.7 BV/h
Methanol wash volume	12.9–13.5 BV
Methanol wash flow rate	8.7–18.9 BV/h (14.6)
UV λ	238 nm

[a]Typical values are shown in bold. BV= bed volume; ACN, acetonitrile.

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part of the main peak were sufficient. During step B, fractions in front of the main peak were collected at intervals of approximately 40 s for a number of injections. Collection of stream samples at 30-s intervals during one injection provided even more detail on the pattern of impurity elution versus time for step B.

Table 2. Preparative Column Operating Parameters During Step B LSHPLC Injections[a]

Chromatographic Parameter	Operating Range
Column condition (10% ACN)	2 BV
Condition flow rate	6.4–14.6
BV/h	
Feed volume	1.4 BV
Feed flow rate	5 BV/h
Post-feed wash (10% ACN with acid)	0.1 BV
Eluent (22% ACN with acid)	6.8 BV
Eluent flow rate	6.4 BV/h
Wash (linear gradient from 22–90% ACN)	3.0 BV
Wash flow rate	14.6 BV/h
Isocratic wash (90% ACN)	3.0 BV
Isocratic wash flow rate	14.6 BV/h
UV λ	240 nm

[a]ACN, acetonitrile; BV, bed volume.

During process development, two short analytical HPLC assays (12 min for step A fractions and 15 min for step B fractions) were used to provide data for fraction selection. These analyses were carried out on a 150 × 4.6 mm Waters Symmetry C^{18} column (Waters Corp., Milford, MA, USA), using a linear gradient of 0.1% vol-% aqueous perchloric acid and acetonitrile (Fisher Scientific). The analytical assays allowed the concentrations of pertinent compounds to be determined, and these results were then tabulated on a spreadsheet. Along with the fraction weights or volumes, these data were used to calculate a predicted impurity profile for combinations of selected fractions. To confirm the accuracy of the calculated impurity composition, composite samples of the proposed combinations were prepared and analyzed. During development, this was done before to actual combination of the fractions in the pilot plant. The data from the analytical assays were then correlated to the UV data generated from the preparatory column detector to develop the criteria that would allow fraction selection to occur without having to wait for the results of the analytical assays.

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RESULTS AND DISCUSSION

The structures of the step A synthetic intermediate [IA] and the step B product, caspofungin [IB], are shown in Figure 1. The starting material for the synthesis, which has been described previously (1), contains a large number of impurities produced during the fermentation that differ only slightly from the main compound. For example, a number of positional isomers are produced during the fermentation. During the synthetic procedure, additional impurities are produced, both from the main compound and from each of the impurities originating from the fermentation.

Step A: Preparative Chromatography

The main purpose of the step A purification is to remove unreacted starting material, which is retained on the column until the methanol wash. Some inorganic salts used in the process are also removed. The approximate location of these and some other elution events are shown in the UV trace from an injection of step A reaction mixture on the preparative column (Fig. 2). A high degree of column performance is not required for this step, and a column pack that yielded a minimum of 12,000 plates/m was acceptable. However, partial rejection of some impurities that elute in front of the main compound is achieved in this step, so fraction selection in this region was monitored closely. Figure 3 shows the time points where fractions were collected for a typical injection. No impurities are removed, to any significant extent, on the tail side of the main peak. The peak

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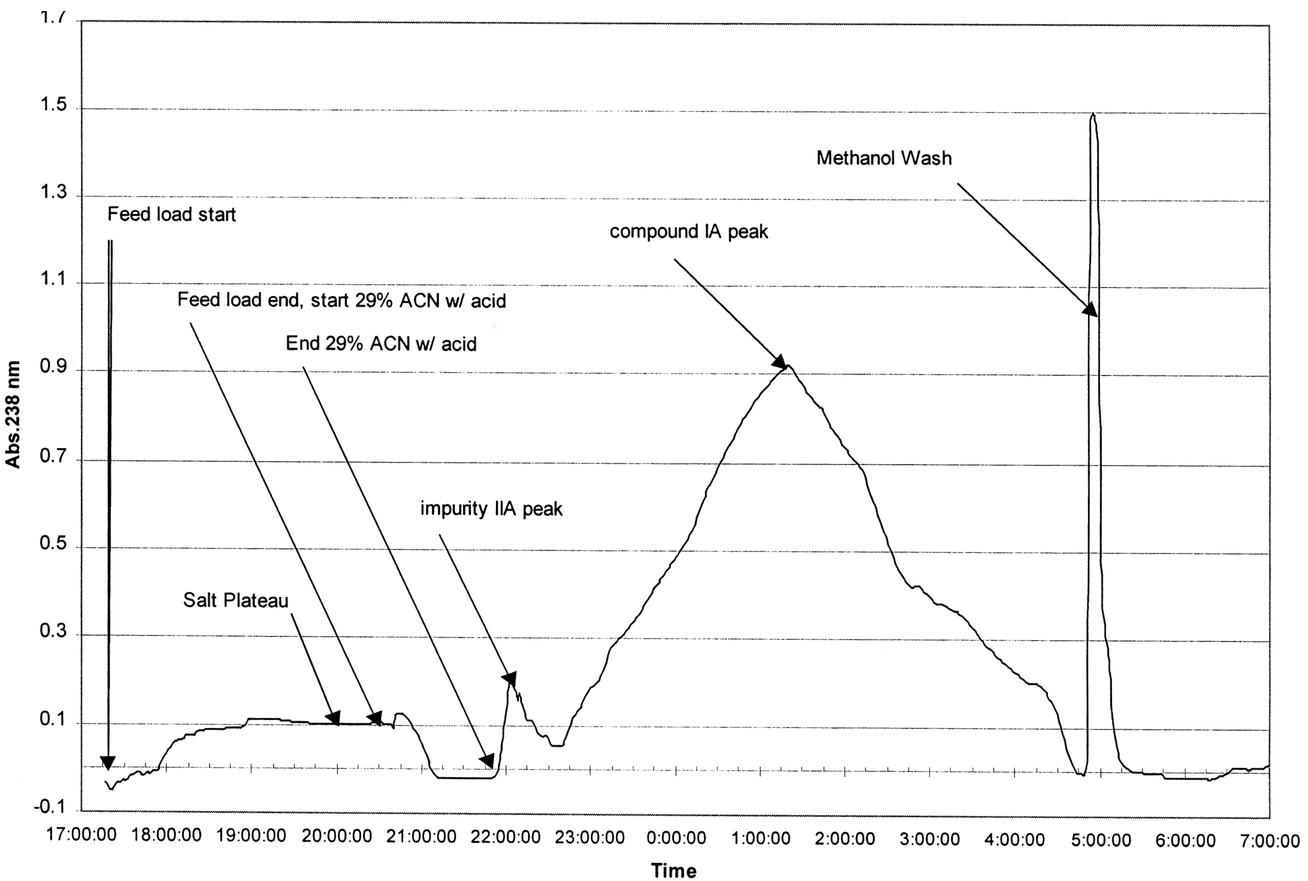

Figure 2. Features of a UV trace of step A eluent from the preparative column, absorbance at 238 nm versus time.

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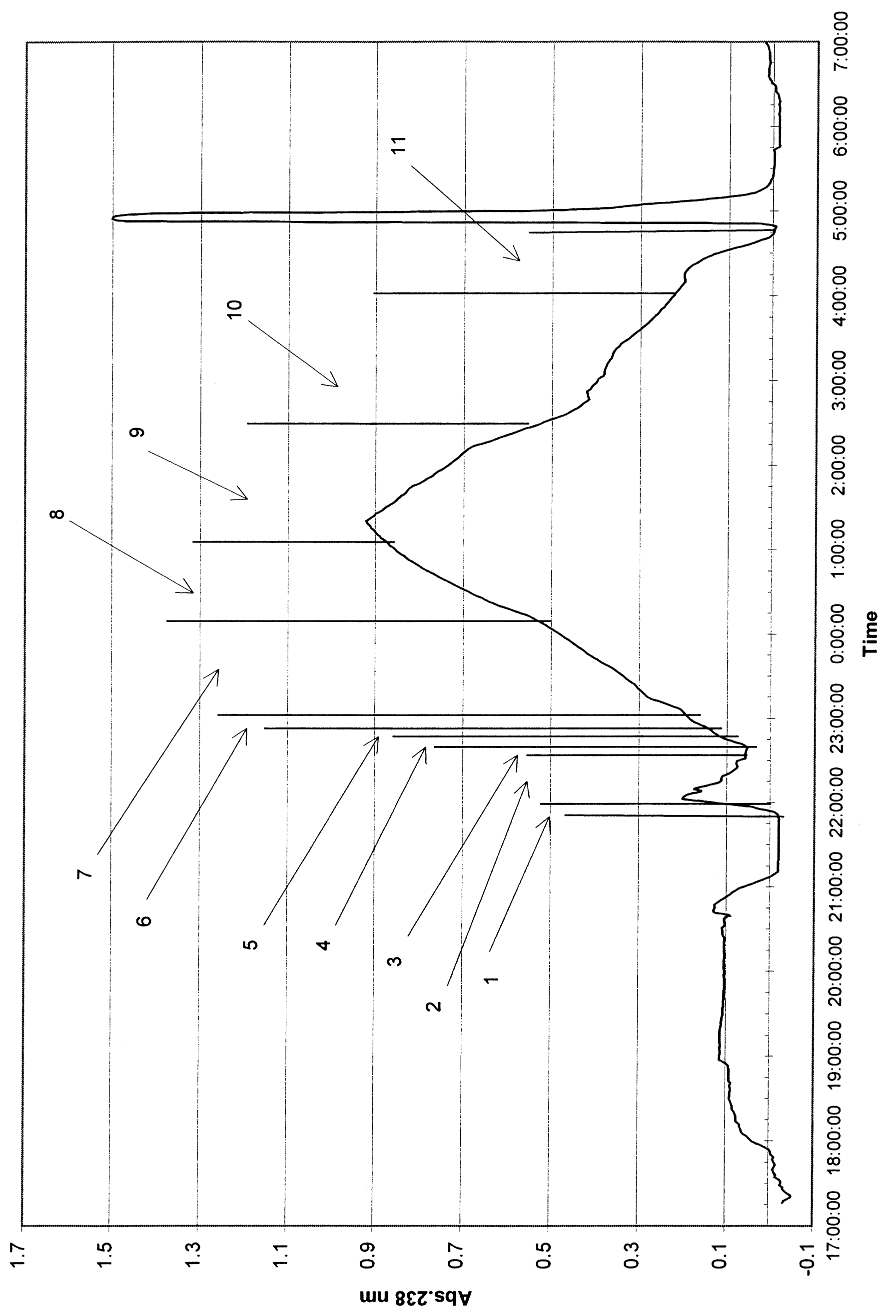

Figure 3. Fraction cut points for an injection of batch during step A LSHPLC.

labeled [IIA] in Figure 2 is an over-reduction impurity. A number of impurities coelute under this peak and tail into the main peak. However, it was found that the levels of the other impurities under peak [IIA] decrease more quickly than [IIA] itself, with one exception, an impurity [IIIA], that is judged to be another over-reduction product, based on its molecular weight. Figure 4 shows how the concentrations of [IIA] and [IIIA] decrease in the fractions. The important feature of their behavior is a crossover point in their rate of decrease. The location of this crossover along the time axis will shift, depending on the initial concentrations in the feed solution and the time points where the fractions are taken. This means that either [IIA] or [IIIA], or both, could end up in significant quantities in the rich cuts and, therefore, both must be monitored during production to determine where rich-cut collection should begin.

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Prediction of Rich-Cut Selection for Step A

There are two important criteria for the process when rich-cut selection is based on only two marker impurities (e.g., [IIA] and [IIIA]). First, the column performance should be reproducible. The reproducibility of the chromatographic features for a series of injections of the same synthetic batch is shown in Figure 5.

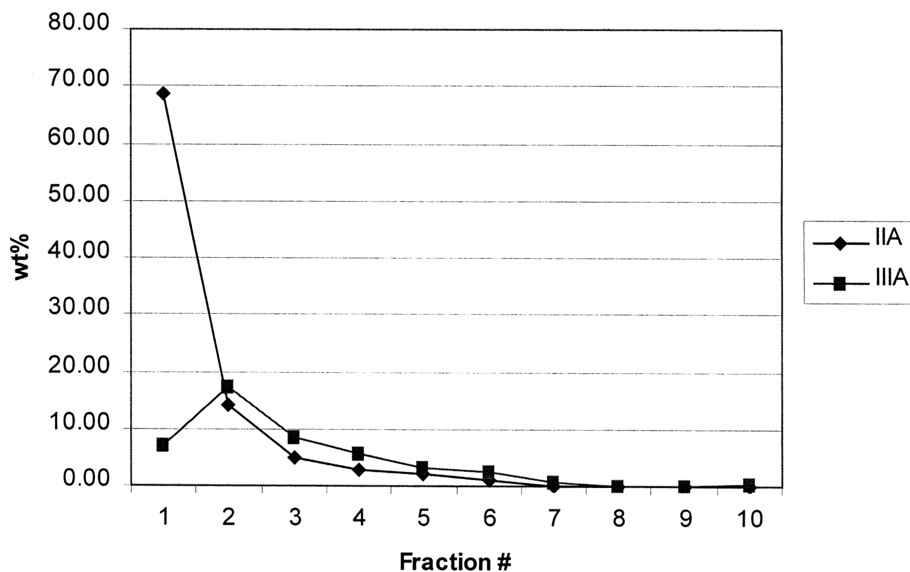

Figure 4. Weight percent of [IIA] and [IIIA] impurities versus fraction number for an injection of batch during step A LSHPLC.

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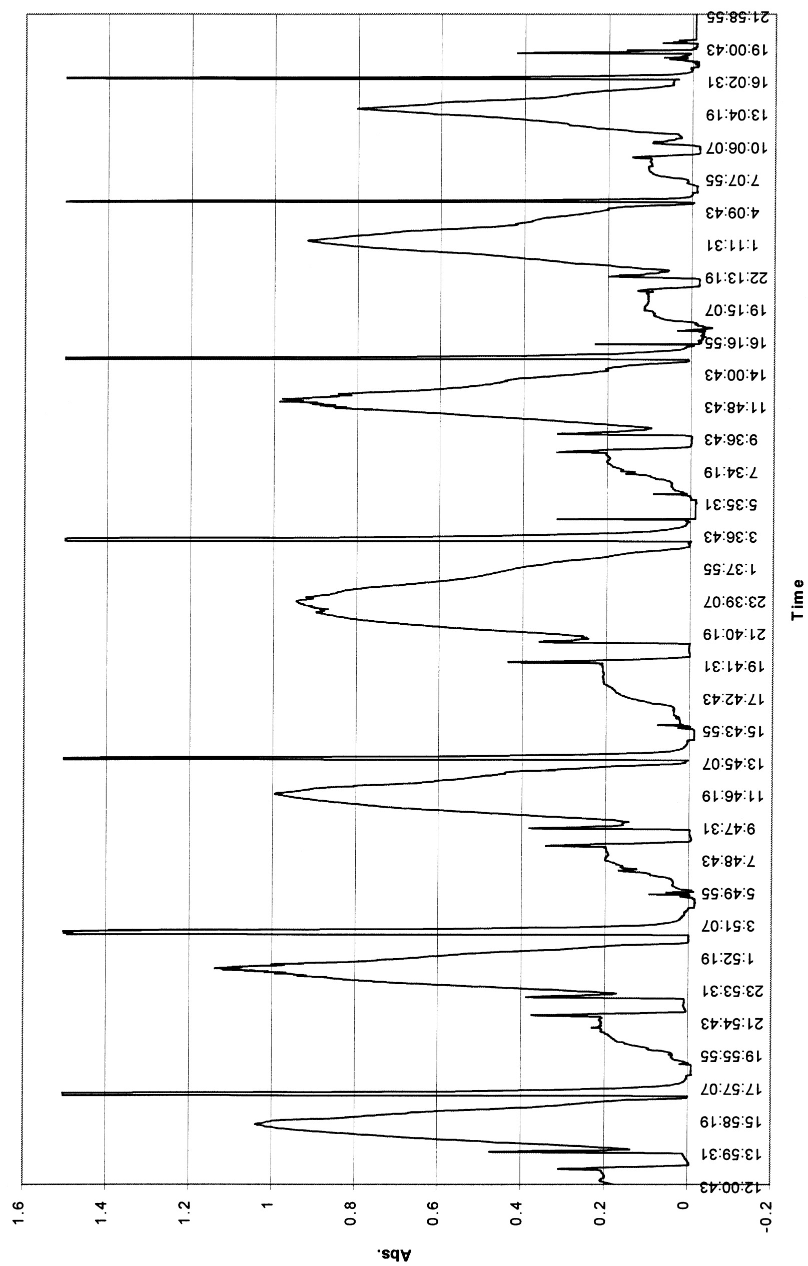

Figure 5. UV trace of preparative column eluent, absorbance at 238 nm versus time, of multiple injections of batch during step A LSHPLC.

Second, the fermentation and synthetic process should be consistent, since the fraction-selection model is based on a particular impurity composition. The fermentation process development team achieved this goal for this process.

The concentrations of [IIA] and [IIIA] in individual fractions were used to predict their concentrations after the fractions were pooled. This is shown in Table 3, along with the levels actually found in a sample of the combined fractions. The predicted level of [IIIA] is typically overestimated due to coelution of other minor components in the short analytical assay. In an individual fraction, the concentration of these components may be high relative to the main compound, but in the final combined sample they do not contribute much to the area counts. A goal for the predicted combined fractions is to end up with each of these two impurities below 0.4%. Our experience with this process has demonstrated that this is a conservative value that allows for small variations in the performance of the preparative column, while maintaining the production of drug substances of consistent quality. Also presented in Table 3 are the results for two other front-eluting impurities ([IVA] and [VA], which are structural analogs derived from the starting material), to show that their levels are well predicted by the analytical assay of individual fractions.

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Development of Fraction Selection Criteria for Step A

The predictions of the final concentrations of [IIA] and [IIIA] in selected rich cuts can be used for determining criteria that specify where rich-cut collection should begin, according to the UV absorbance of the preparative column detector. As an example, Table 3 also shows how the composition of the final combined rich cuts would have changed if the start of fraction collection had

Table 3. Predicted and Actual Results of Key Impurities for Various Combinations of Fractions from Seven Injections of Batch During Step A LSHPLC[a]

	[IIA]	[IIIA]	[IVA]	[VA]
Found, actual combined fractions	0.17	0.22	0.22	0.19
Predicted for combined fractions	0.14	0.29	0.19	0.21
Predicted UV/UV_{max} main >0.4	0.10	0.26	0.17	0.19
Predicted UV/UV_{max} main >0.45	0.09	0.24	0.17	0.19
Predicted UV/UV_{max} main >0.5	0.09	0.23	0.16	0.18

[a]The table predictions given are weight percent. The actual analysis results are area percent. The response factors of the compounds under consideration are the same; therefore, the weight percent and area percent results are directly comparable.

shifted based on different UV detector readings. This was done by comparing the UV trace of the preparative column detector to the results of the analytical HPLC area percent analyses of the fractions collected. Instead of an absolute absorbance value, a ratio of the absorbance to the absorbance maximum of the main peak is used. This allows the information to be transferred to other LSHPLC setups with different detectors, which may have different path lengths or different intensity lamps. The predicted values in Table 3 are approximate since they represent the sum of data from multiple injections, and the collection of the actual fractions for a particular injection may not have started exactly at the UV value used in the table. For example, if collection of a fraction was started at a UV/UV_{max} of 0.42, it was included in the prediction for UV/UV_{max} >0.4; if it started at UV/UV_{max} of 0.43, it was included in the prediction for UV/UV_{max} >0.45. For this reason, the procedure is most accurate when smaller fractions are collected. The predicted composition for the fractions that were actually combined was closest to the UV/UV_{max} >0.4 ratio. UV/UV_{max} ratios below this value resulted in predicted impurity levels that were above target goals. Using the value of UV/UV_{max} >0.5 would have resulted in a slight increase in purity, but would also have resulted in a predicted decrease in the yield of approximately 2.2%. The final selection of the UV ratio to be used is based on the trade-off between acceptable purity and yield loss.

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Step B: Preparative Chromatography

For the step B preparative chromatography, the separation is much more demanding since stricter product specifications must be met at this stage, and the separation consists of removal of impurities that are nearly identical structural analogs. For this reason, greater attention was placed on correlating the results of tracer tests to column performance. An initial test at the beginning of batch 1 (B1) showed excellent column performance, yielding approximately 30,000 plates/m. Analytical results for the fractions from the first few injections of B2 indicated a decline in column performance and poor resolution of analog impurities, and another tracer test was performed. Unlike step A for which a minimum plate count of 12,000 plates/m was acceptable, a plate count of <15,000 plates/m was found to be unacceptable during this step. The column was repacked, and a tracer test was performed at the beginning of each subsequent batch to check for acceptable performance.

A typical chromatogram for the preparative column eluent from an injection during step B is shown in Figure 6, and the key features of the chromatogram are labeled. During the step B chromatography, two key impurities can serve as markers for rich-cut selection, one at the front and one at the back of the main

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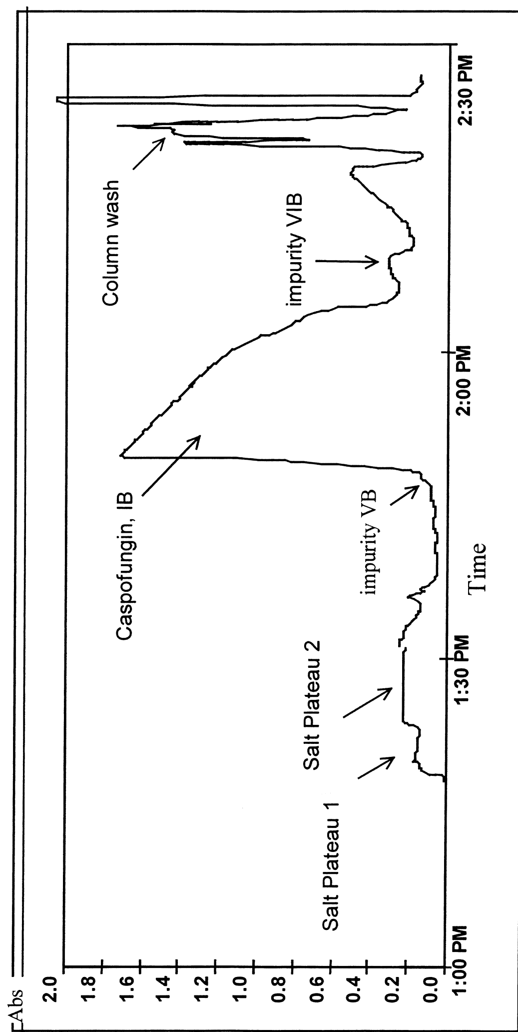

Figure 6. Features of a UV trace of preparative column eluent during the step B LSHPLC.

peak. On the front side of the main peak, the key impurity is a structural impurity [VB] that carries through from Step A, and on the tail side it is a different over-reduction product [VIB]. Although only two impurity peaks are labeled, there are actually a number of coeluting impurities under each impurity "peak." Typical fraction collection points are shown in Figure 7. Fractions 1, 2, and 3 are taken only a few minutes apart since this is the location where the beginning of a single rich cut would occur, and, hence, where the change in impurity composition will have the greatest impact on the quality of the final product. A series of injections on a compressed time scale shown in Figure 8 demonstrated excellent reproducibility. Because of the more stringent purity requirements for this step, stream samples of eluent for one injection were collected at closely spaced intervals obtain to obtain a more detailed impurity profile. The corresponding concentration profile for selected impurities in the stream samples is shown in Figure 9. Stream samples 1–15 and 27–31 were taken at ½-min intervals, and the others were taken at 1-min intervals. Note that significant changes in the levels of impurities occur in less than 4 min, much less than the turnaround time for the available analytical HPLC assay, making an on-line HPLC analysis impractical.

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Development of Fraction Selection Criteria for Step B

The results of HPLC area-percent analyses for the marker compounds in the actual pooled rich cuts are shown in Table 4, along with the predicted weight percent for various combinations of fractions. The upper allowable limit (target maximum) for each impurity listed is also given. The level of coeluting impurities will automatically be below the target maximum when the values for the key marker compounds are met. As an example, the values for two impurities that carry through from step A ([IIB] and [IVB]) that coelute under [VB] are shown in Table 4. As with step A, these results can be applied to the UV absorbance data obtained from the preparative column to establish criteria for determining where rich-cut selection should begin and end; these are shown in Table 5. In this step, absolute absorbance values were used, rather than ratios, since it was established that the same detector would be used throughout. The results in Table 4 show that the actual combination of fractions 3 and 4 was conservative. The addition of fraction 2 to the pool would not have resulted in a significant increase in impurity levels. The addition of fraction 5 from the tail side would probably have resulted in an excess level of impurity [VIB]. However, it appears that at least part of the beginning of fraction 5 could have been included without exceeding the target level for this impurity. The main factor affecting the decision then becomes yield. The concentration of caspofungin in those additional fractions is so low that their addition to the pool does not add any value to the process.

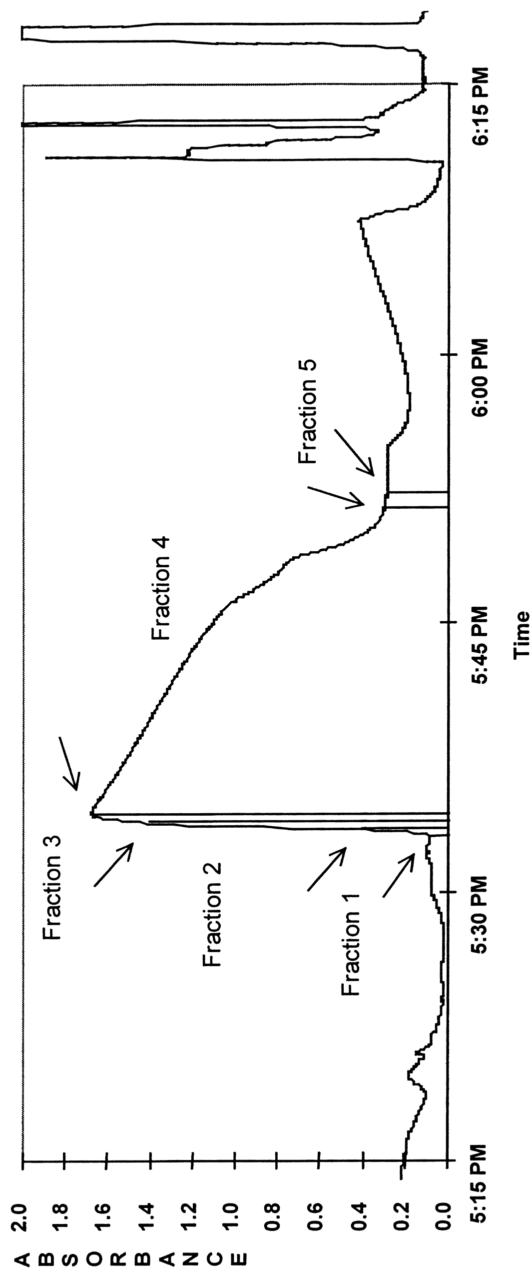

Figure 7. Fraction cut points for an injection of batch during step B LSHPLC.

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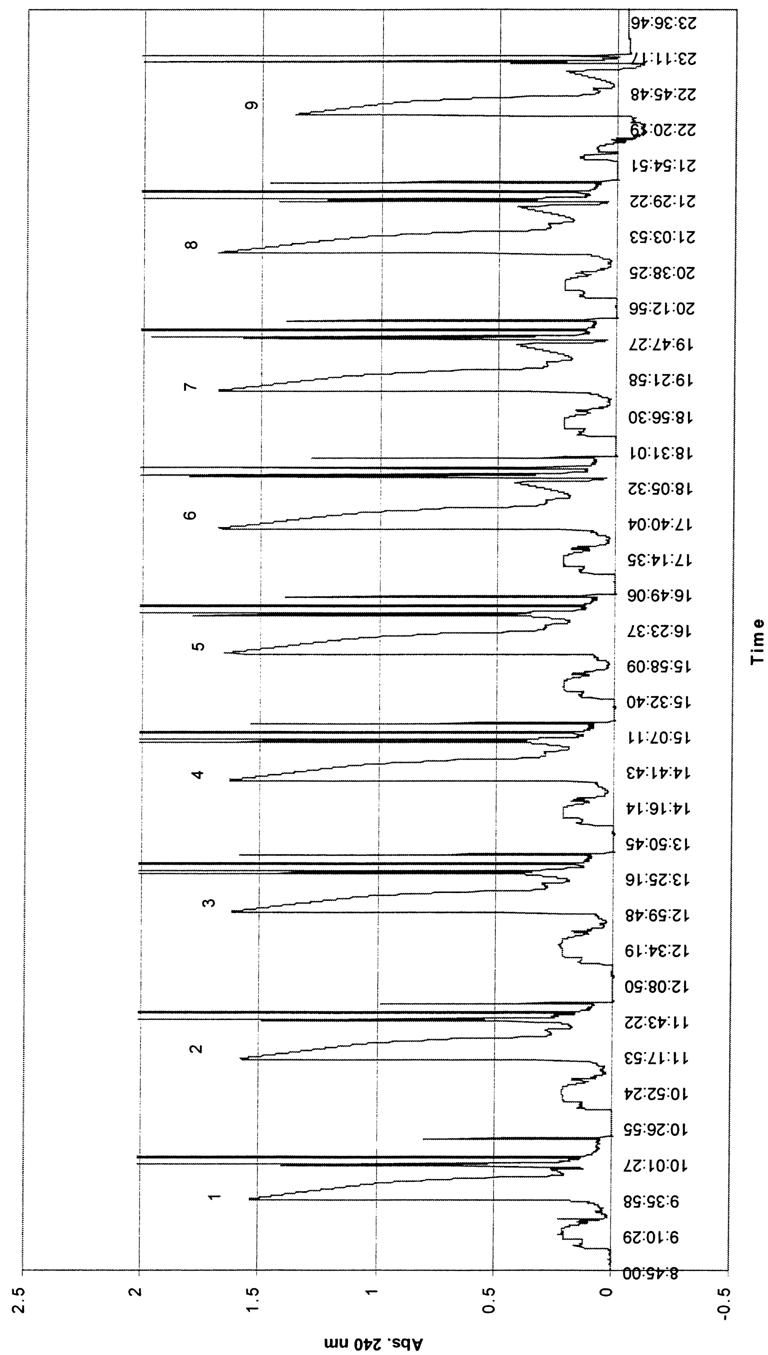

Figure 8. UV trace of preparative column eluent, absorbance at 240 nm versus time, of multiple injections of batch during step B LSHPLC.

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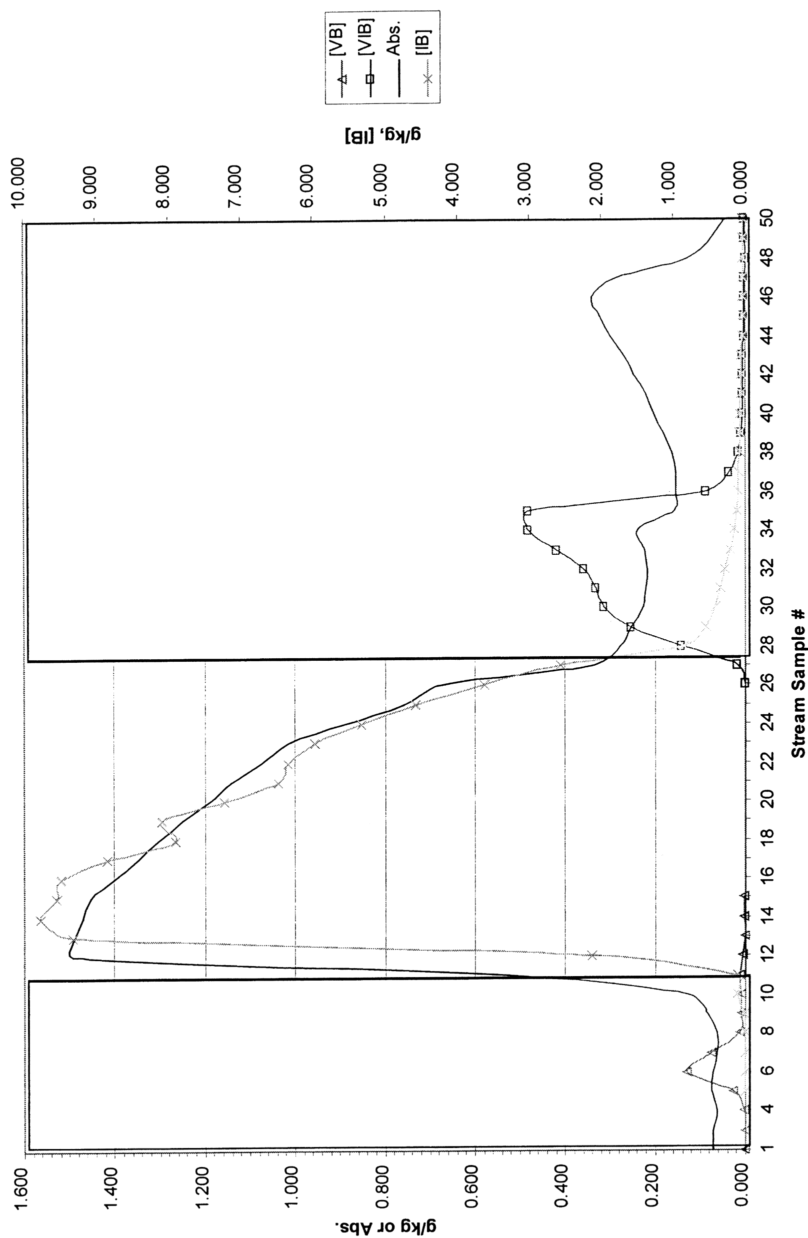

Figure 9. Concentration of selected compounds in stream samples, and preparatory column absorbance trace (240 nm) during step B LSHPLC. Vertical lines show where the rich cut would be using UV criteria. Concentration (g/kg) of minor impurities is shown on the left *y*-axis; concentration (g/kg) of the caspofungin acetate [IB] peak is shown on the right *y*-axis.

Table 4. Predicted and Actual Results of Key Impurities for Various Combinations of Fractions from Nine Injections of Batch During Step B LSHPLC[a]

	[IIB]	[IVB]	[VB]	[VIB]
Target maximum	0.2	0.2	0.1	0.4
Actual found, fr. 3 + 4[b]	0.00	0.00	0.03	0.23
Predicted, fr. 3+4	0.08	0.00	0.03	0.24
Predicted, fr. 2+3+4	0.08	0.00	0.04	0.23
Predicted, fr. 3+4+5	0.08	0.00	0.03	0.45
Predicted, fr. 2+3+4+5+6	0.08	0.00	0.04	0.65
Predicted, fr. 1+2+3+4	0.10	0.00	0.11	0.24

[a]The table predictions given are weight percent. The actual analysis results are area percent. The response factors of the compounds under consideration are the same; therefore, the weight percent and area percent results are directly comparable.
[b]A longer analytical assay was used for this sample that provides more resolution between [IIB] and nearby impurities than the short analytical assay used for the column fractions (fr).

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CONCLUSION

A resolution test (using a simple organic compound) provides preliminary information about the column each time the column is repacked or at times when there is a suspected problem. However, a full-scale injection of batch material, combined with analysis of fractions, is essential for evaluation of the column performance and development of UV absorbance criteria for selection of fractions. Knowledge of the impurity profile of the feed solution is also important, since a large change in the composition of impurities could theoretically affect the reso-

Table 5. Cut Strategy Criteria for the Processing of Step B Injections

Step No.	Criteria to Move to Next Step	Action
1	Volume > 0.9 BV	Stop sample pump, start 10%
2	Volume > 1.0 BV	End 10%, start 22%
3	Volume >3.6 BV and UV >0.09 AU	Start fraction 1
4	Volume >3.6 BV and UV > 0.4 AU	Start fraction 2
5	Volume >3.6 BV and UV > 1.4 AU	Start fraction 3
6	Volume >3.6 BV and UV>1.8 AU or Volume >3.6 BV and UV slope < 0	Start fraction 4
7	Volume >3.6 BV and UV < 0.33	Start fraction 5
8	Volume >3.6 BV and UV < 0.30	Start fraction 6

lution on the preparative column. The rich-cut selection criteria are determined by the UV absorption spectra of a batch having a particular composition and, therefore, will not be applicable if levels of impurities vary in an unknown way. Consistent operating conditions, including reproducible column performance, are of prime importance in automated fraction collection. However, the use of conservative criteria (both in acceptance levels for impurities and in the value of the UV ratio for selection of fractions) should allow material of acceptable purity to be collected, even if there is some deviation in the performance of the preparative column. A model for automated fraction collection, based on the UV criteria and volume of eluent, should make the large-scale preparative HPLC purification procedure more reproducible and economical, even though some yield may be sacrificed in order to meet target purity levels.

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