

Effect of Mobile Phase Conditions on the Purification of Vancomycin Peptide

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Vancomycin is a branched tricyclic glycosylated nonribosomal peptide produced by the fermentation of *Amycolatopsis orientalis*, and it is used in the treatment of infections caused by Gram-positive bacteria.

A typical purification protocol of vancomycin includes **reversed phase chromatography**. This study examines the effect of mobile phase conditions, including pH, on the reversed phase purification of a crude vancomycin broth.

Both **silica and polymeric reversed phase chromatography packings** are evaluated for their effectiveness in providing high yield and purity.

Results demonstrate that **polymeric chromatography packings** operated under basic pH conditions are **effective and economical** for the purification of vancomycin.

Process Development

Process development for the vancomycin purification was done in several phases:

1. Initial Low-Load Experiments

Low-load experiments were conducted with several different reversed phase packing materials under several different mobile phase conditions. These experiments allowed us to determine which packing materials and conditions would be used for preparative-load purifications.

2. Dynamic Capacity Measurements

In conjunction with the low-load experiments, a series of dynamic capacity measurements were determined. From these results, we were able to set appropriate loading levels for the preparative-load purifications.

3. Preparative-Load Experiments

Based on the two previous sets of experiments, several preparative purifications were conducted. These included purifications that used both untreated and decolorized broth.

4. Decolorization Experiments

Decolorization of the crude filtered vancomycin broth was done using several different anion exchange resins using different pHs. Based on these initial experiments, broth was decolorized prior to the preparative RPC purification of the vancomycin broth.

Low-Load Experiments

Low-load experiments were conducted under the following conditions:

- Sample: vancomycin broth, expressed from yeast and filtered with 0.7mm filter
- Loading: 2.5mg of vancomycin, influent purity of 34%
- Column: 1.0cm I.D. x 25cm L, packing material listed in Table 1
- Mobile Phase: A: listed in Table 1
- B: acetonitrile
- Gradient: 7.5% to 30% B in 150 minutes
- Flow rate: 1mL/min
- Detection: UV @ 280nm
- Fractions: 1mL

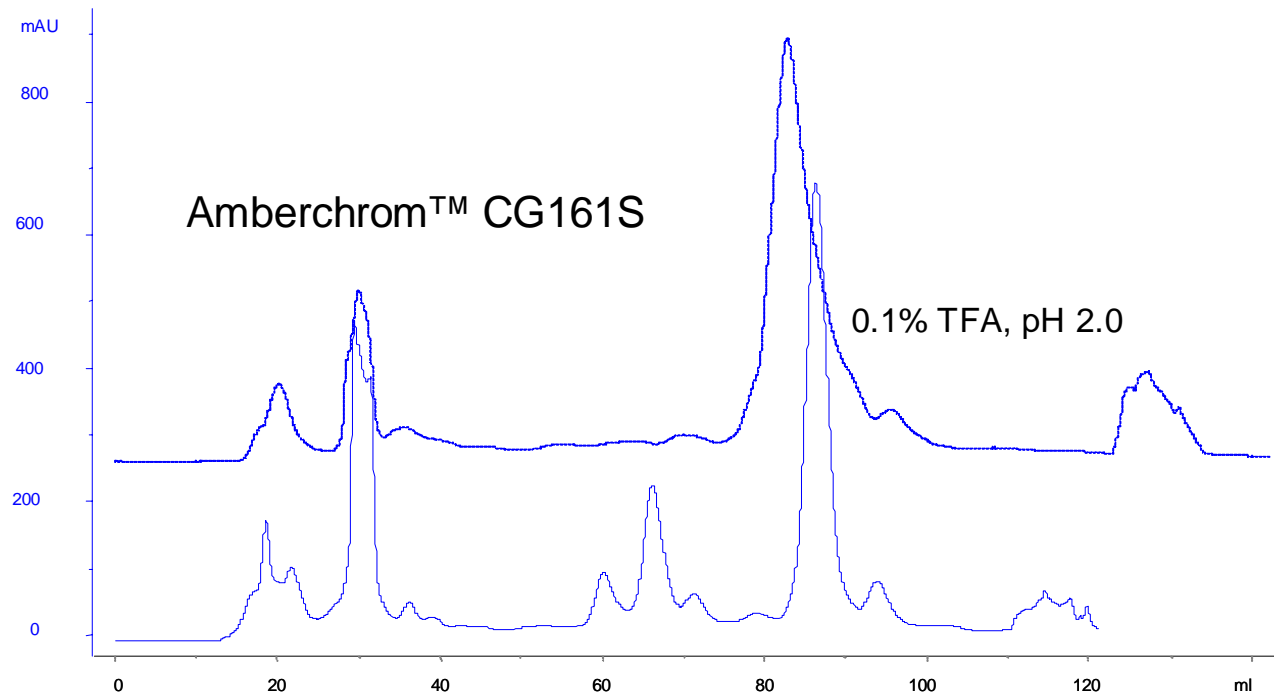
As shown in Table 1 and Figure 1, mobile phase conditions and pH had a dramatic effect on the yield and purity of vancomycin. Additionally, the silica columns (Vydac C4, YMC C4 and Kromasil™ C8) generally performed better than the polystyrenic Amberchrom™ columns at low pH. At basic pH, however, the polystyrenic columns performed as well, or better, than the silica columns. Not surprisingly, the small particle size resins performed better than the larger resins. Operating the purifications with the ammonium acetate provided the additional benefit of a volatile salt and left the peptide in the desirable acetate form. Finally, dynamic capacities (shown in the next section) were highest at basic pHs. Therefore, due to the stability of the polystyrenic resins at high pH, all further work was performed with the polystyrenic Amberchrom™ resins.

Low-Load Experiments

Table 1
Low-load Experiments – Effect of Mobile Phase and pH

Resin	Conditions	Total Recovery	85% Purity Recovery	90% Purity Recovery	95% Purity Recovery
Amberchrom HPR10, 10 micron	0.1% TFA, pH 2.0	84%	71%	48%	37%
Amberchrom HPR10, 10 micron	50mM tris, pH 8.0	94%	92%	92%	84%
Amberchrom HPR10, 10 micron	50mM acetate, pH 8.5	94%	88%	88%	79%
Amberchrom XT20	0.1% TFA, pH 2.0	97%	81%	65%	40%
Amberchrom XT20	50mM tris, pH 8.0	94%	88%	84%	74%
Amberchrom XT20	50mM acetate, pH 8.5	87%	80%	80%	67%
Amberchrom CG161S, 35 micron	0.1% TFA, pH 2.0	100%	69%	56%	0%
Amberchrom CG161S, 35 micron	50mM tris, pH 8.0	96%	86%	86%	69%
Amberchrom CG161S, 35 micron	50mM acetate, pH 8.5	91%	84%	80%	69%
Vydac C4, 10 micron	0.1% TFA, pH 2.0	100%	97%	75%	45%
Vydac C4, 10 micron	50mM tris, pH 8.0	98%	90%	70%	26%
Vydac C4, 10 micron	50mM acetate, pH 8.5	100%	96%	76%	38%
YMC C4, 10 micron	0.1% TFA, pH 2.0	100%	100%	96%	96%
YMC C4, 10 micron	50mM tris, pH 8.0	97%	92%	92%	75%
YMC C4, 10 micron	50mM acetate, pH 8.5	97%	89%	86%	50%
Kromasil C8, 10 micron	0.1% TFA, pH 2.0	100%	98%	92%	79%
Kromasil C8, 10 micron	50mM tris, pH 8.0	99%	97%	87%	69%
Kromasil C8, 10 micron	50mM acetate, pH 8.5	97%	96%	88%	78%

Low-Load Experiments



Dynamic Capacity Measurements

Dynamic capacities for vancomycin were determined using the following conditions:

- Sample: vancomycin hydrochloride, Sigma Aldrich V2002
- Column: Amberchrom CG161S, 1.0cm I.D. x 25cm L
- Flow rate: 2 mL/min
- Mobile Phase: listed on table + 7.5% acetonitrile
- Detection: UV @ 280nm

As shown in Table 2, pH has a profound impact on the dynamic binding of vancomycin on the polystyrenic reversed phase resin, Amberchrom™ CG161S. Basic pH conditions improve capacity by a factor of three to four over acidic conditions. Although not shown, capacity measurements with other packing materials, whether silica or polystyrenic, indicated the same binding capacity differences. In addition, there does appear to be a slight effect of the mobile phase itself. Capacities obtained at pH 8 using Tris-HCl were slightly higher than those obtained using ammonium acetate at pH 8. This may be a function of the salt form of the vancomycin. Finally, increasing the concentration of buffering salt (from 50mM to 100mM) did not appear to affect on the binding capacity.

Dynamic Capacity Measurements

Table 2
Amberchrom™ CG161S Dynamic Capacities

Conditions	1% Capacity (mg/mL)	Total Capacity (mg/mL)
0.1% TFA, pH 2.0	20	21
50mM Ammonium Acetate, pH 5.0	25	29
50mM Ammonium Acetate, pH 7.0	41	56
50mM Ammonium Acetate, pH 8.0	89	93
50mM Tris-HCl, pH 8.0	107	110
50mM Ammonium Acetate, pH 8.5	85	93
100mM Ammonium Acetate, pH 8.5	91	94

All loading mobile phases contained 7.5% acetonitrile

Preparative-Load Purification

Preparative purifications of vancomycin were done under the following conditions:

- **Sample:** crude vancomycin broth, filtered with 0.7mm filter
- **Loading:** 366mg of vancomycin on Amberchrom™ CG161S column
288mg of vancomycin on Amberchrom™ HPR10 column
- **Column:** 1.0cm I.D. x 25cm L
- **Mobile Phase:** A: 50mM ammonium acetate, pH 8.5
B: acetonitrile
- **Gradient:** 7.5% to 30% B in 360 minutes
- **Detection:** UV @ 280nm

Figures 2 and 3 show the results for the two-step preparative purification of vancomycin using Amberchrom™ CG161S and Amberchrom™ HPR10. The loading on the Amberchrom™ CG161S column was 18.6mg/mL and the yield and purity were 79% and 82%, respectively. The initial purity of the feed was 34%.

The Amberchrom™ CG161S pool was diluted 50/50 with 50mM ammonium acetate in order to adjust the feed concentration so that the acetonitrile concentration was approximately 7.5%. This diluted pool was then loaded onto the Amberchrom™ HPR10 column. This second step gave a 61% yield of 90% pure vancomycin for a total overall yield of 48%.

Preparative-Load Purification

Figure 2
Vancomycin Preparative Chromatograms

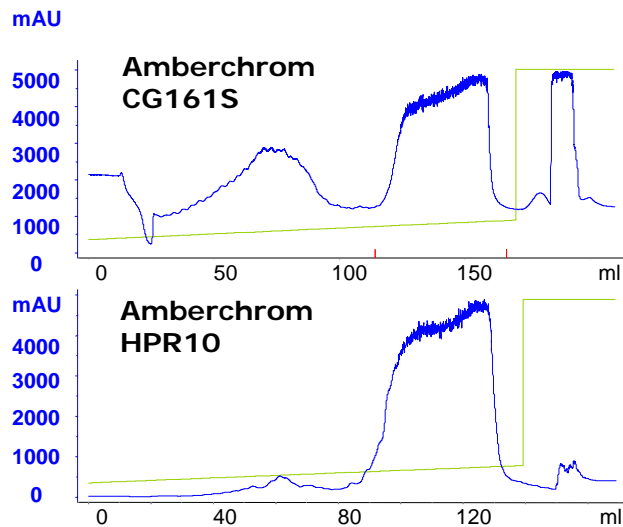
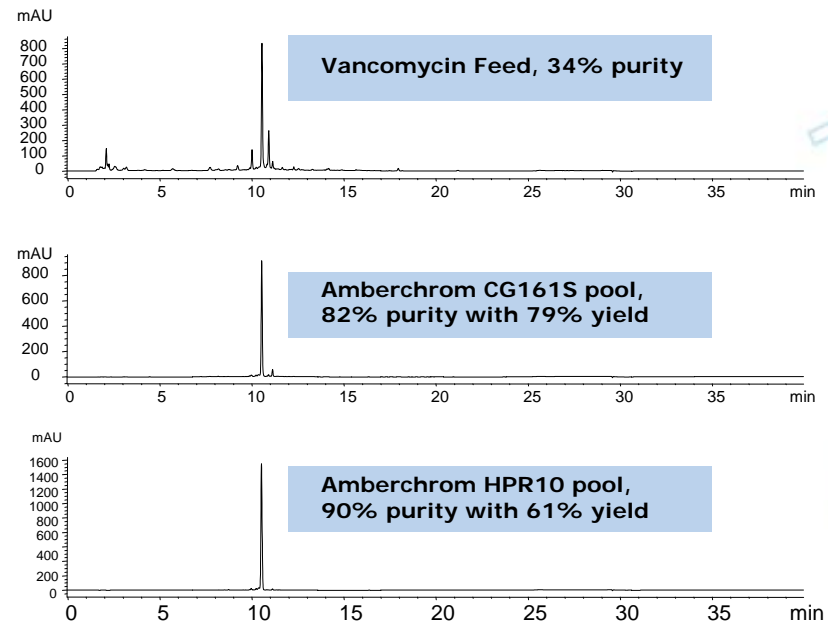


Figure 3
Vancomycin Purification – Fraction Analysis



Decolorization Experiments

Batch decolorization experiments were conducted with the crude filtered vancomycin broth by adding 1g (based on dry weight) of Amberlite™ FPA resin to 20mL of broth, and shaking the contents for four hours. The pH of the broth was adjusted by titrating with ammonium hydroxide. Color removal was assessed spectrophotometrically at 410nm, and vancomycin recovery was assayed via reversed phase HPLC.

Table 3 presents the color removal data for the different resins and pHs, and Table 4 presents the corresponding vancomycin recovery for the different resins and pHs. All of the weak anion exchange resins; Amberlite™ FPA51, Amberlite™ FPA53, Amberlite™ FPA54, and Amberlite™ FPA55, provided excellent decolorization. However, vancomycin recovery at all pHs with these resins was poor.

Amberlite™ FPA90 Cl and Amberlite™ FPA98 Cl at near-neutral or neutral pHs were determined to be **the best candidates for vancomycin broth decolorization.**

Decolorization Experiments

Table 3
Color Removal with Amberlite™ FPA Resins

Resin	pH 2.5	pH 5.0	pH 7.0	pH 8.0	pH 8.5
Amberlite FPA40Cl	40%	68%	85%	85%	85%
Amberlite FPA90Cl	28%	58%	79%	80%	83%
Amberlite FPA98Cl	23%	48%	71%	72%	75%
Amberlite FPA51	75%	83%	85%	84%	84%
Amberlite FPA53	68%	71%	68%	65%	64%
Amberlite FPA54	71%	75%	74%	70%	69%
Amberlite FPA55	70%	76%	74%	71%	72%

Table 4
Vancomycin Recovery with Amberlite™ FPA Resins

Resin	pH 2.5	pH 5.0	pH 7.0	pH 8.0	pH 8.5
Amberlite FPA40Cl	84%	79%	52%	23%	14%
Amberlite FPA90Cl	93%	91%	77%	57%	39%
Amberlite FPA98Cl	94%	92%	83%	70%	59%
Amberlite FPA51	31%	26%	21%	20%	19%
Amberlite FPA53	21%	30%	35%	36%	39%
Amberlite FPA54	59%	53%	48%	48%	47%
Amberlite FPA55	23%	26%	28%	30%	30%

Amberlite™ FPA98Cl - Cycling

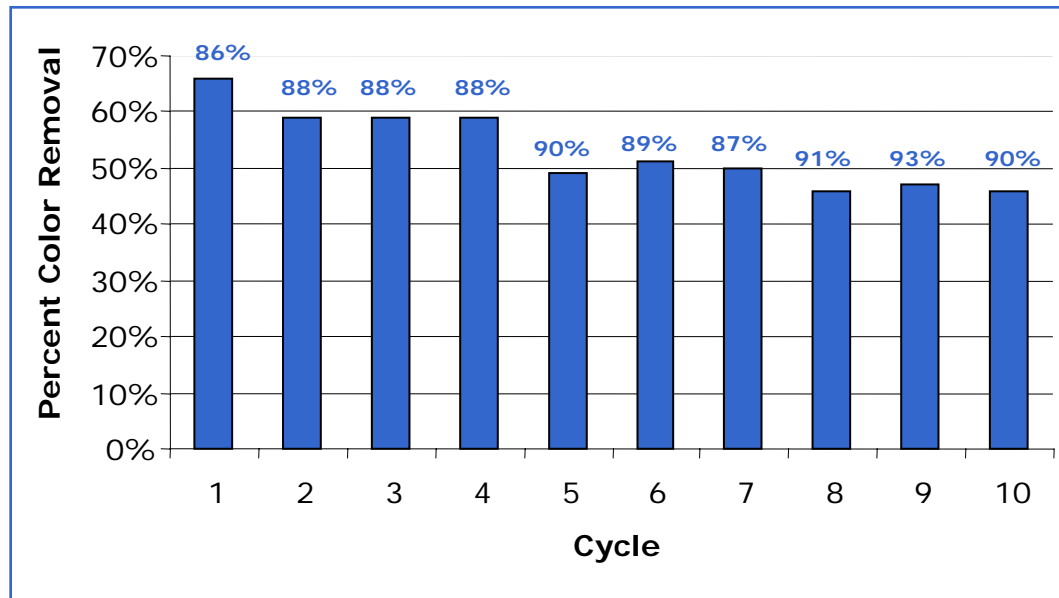
A sample of **Amberlite™ FPA98Cl** was subjected to ten decolorization cycles by weighing out 10g (dry weight) of resin into 200mL of crude filtered vancomycin broth and shaking the contents for two hours on an orbital shaker. The resin was then recovered from the treated broth, and the treated broth was assayed at 410nm for color removal determination and by reversed phase HPLC to determine vancomycin recovery. The recovered resin was then weighed back into a bottle and a fresh 200mL of crude filtered vancomycin broth was added and shaken for two hours. This was repeated for a total of ten cycles.

As shown in Figure 4, **the color removal was still fairly effective after ten cycles and vancomycin recovery was good throughout the ten cycles.** It must be emphasized that the Amberlite FPA98Cl was not regenerated in any way during this cycling study. Amberlite FPA98Cl can be effectively regenerated with 0.1N HCl and then re-equilibrated with a 10% NaCl solution in 0.5% NaOH.

Amberlite™ FPA98CI - Cycling

Figure 4
Amberlite™ FPA98CI – Cycling Results

Percentages in blue represent recovery of vancomycin



Final Preparative Purifications

Based on the decolorization results, several further preparative purifications were conducted at loading levels of 20-25g/L. Initially the purifications were done with no further pH adjustment of the vancomycin broth after decolorization. The results indicated that reversed purifications conducted at **pH 5.0 and pH 7.0** provided relatively low purities (<**75%**). However, if the pH of the decolored broth was adjusted to a more basic **pH of 8.5**, the purity levels improved (>**85%**). These purity levels could be achieved even with a single reversed step using a larger particle size resin, such as **Amberchrom CG161M**. Some of the results are summarized below in Table 5.

Final Preparative Purifications

Table 5
Final Preparative Purifications

Reversed Phase Resin	Conditions	Final Purity	RPC Yield	Overall Process Yield
Amberchrom™ CG161S, 35 m	broth decolorized with Amberlite™ FPA90Cl at pH 5.0 RPC purification run at pH 5.0	70%	68%	62%
Amberchrom™ CG161M, 75 m	broth decolorized with Amberlite™ FPA90Cl at pH 5.0 then pH adjusted to 8.5 RPC purification run at pH 8.5	87%	71%	65%

CONCLUSIONS

- High pH mobile phase conditions improved the yield and purity of vancomycin, particularly on **polystyrenic Amberchrom™ resins**. Additionally, high pH conditions increased the binding capacity of vancomycin to reversed phase packing materials.
- An effective, two-step reversed process was developed for the purification of crude vancomycin. **An overall yield of 48% with 90% purity was achieved.**
- Decolorization of crude filtered vancomycin improved the purification of the vancomycin with subsequent reversed phase chromatography. The decolorization also diminished fouling of the reversed phase packing material, and allowed an effective single reversed phase step to achieve **purity levels of 87% with an overall process yield of 65%.**
- **Amberlite™ FPA resins** were extremely efficient for the removal of color from the crude fermentation broth. The resins could be used for multiple cycles with **effective decolorization** and **high recoveries of the vancomycin product.**