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# Quantitation of vancomycin and its crystalline degradation product (CDP-1) in human serum by high performance liquid chromatography

Dorothy W. Backes, Hoda I. Aboleneen *, Janice A. Simpson

*Analytical Research, Diagnostics Division, Abbott Laboratories, D-90T, AP-20, 100 Abbott Park Road, Abbott Park, IL 60064-3502, USA*

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**Abstract**

The delayed clearance of vancomycin results in accumulation of vancomycin crystalline degradation product, CDP-1, in the bodies of renally impaired patients. The 2 isomers, CDP-1-M (major) and CDP-1-m (minor), of CDP-1 are antibiotically inactive but cross-react with some immunoassays that use polyclonal antibodies resulting in falsely elevated results. A high performance liquid chromatographic (HPLC) method was developed to quantitate vancomycin and CDP-1 in the serum of renal patients. After solid phase extraction of 200 μl serum, the separation of vancomycin, the 2 isomers of CDP-1 and the internal standard (cefazolin) was accomplished by gradient HPLC on a reversed phase C18 column with detection at 210 nm. Linearity was established from 1 to 25 and 25 to 100 μg $ml^{-1}$ vancomycin and 1 to 25 μg $ml^{-1}$ CDP-1. Coefficients of variation for vancomycin and CDP-1 were 3.3–8.6% ($n = 10$) and 2.8–5.2% ($n = 8$). 

*Keywords:* Vancomycin; CDP-1; Reversed phase high performance liquid chromatography; Renal patients

## 1. Introduction

Vancomycin is an amphoteric glycopeptide antibiotic that is active against gram-positive bacteria, including methicillin-resistant staphlococci [1]. Monitoring of serum levels is necessary to ensure adequate therapeutic concentrations while avoiding toxic accumulations [2,3].

Serum half-life is between 5 and 11 h in normal patients, with the majority of an injected dose of vancomycin excreted unchanged in the urine [4,5]. Renal impairment increases vancomycin half-life, which, in dialysis patients, may extend to 5 days or longer [6,7].

Because of speed and ease of operation, immunoassay methods are extensively used to monitor vancomycin in patient serum. During a pharmacokinetics study [8] of vancomycin in dialysis patients, a discrepancy was noted between the original fluorescence polarization immunoassay

* Corrresponding author. Tel.: +1 847 9375179; fax: +1 847 9387922; e-mail: Hoda.Aboleneen@add.sww.abbott.com

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(FPIA) utilizing a polyclonal antibody and high performance liquid chromatography (HPLC) quantitations. It was considered possible that overestimation of vancomycin by FPIA was caused by accumulation of cross-reacting degradation products formed during long periods of time at body temperature. In vitro studies at 37°C showed progressive loss of vancomycin, with FPIA analysis giving an increasingly high bias relative to HPLC determinations over a 10 day period.

In another study [9], it was speculated that the cross-reacting substance was the crystalline degradation product, CDP-1, which is structurally similar to vancomycin, but biologically inactive [10]. Serum, dialysis fluid and saline containing vancomycin were incubated at 37°C and degradation products separated by HPLC. Two of the resulting peaks were identified as CDP-1-M and -m (major and minor isomers).

Papp and Sharpe reported a study which involved measurement of vancomycin in preterm neonates by polyclonal FPIA and HPLC [11]. Overestimation of vancomycin by the FPIA assay was attributed to CDP-1 that had accumulated in the serum of these neonatal patients due to their rather poor renal function.

A number of relatively rapid HPLC vancomycin assays have been developed using protein precipitation [12–14], protein precipitation followed by extraction into an organic solvent [15–19] or solid phase extraction [20–23] for sample preparation. A method which uses automated column-switching with no sample pretreatment has also been reported [24]. However, none of these methods separated or quantitated CDP-1.

In a quantitative method for vancomycin and CDP-1 [25,26], sample preparation involved protein precipitation, solvent extraction to remove lipids and filtration. Sample size was 1 ml. Vitamin $B_{12}$ was used as internal standard and extractions were carried out in darkness at 4°C. Gradient HPLC separation was followed by dual wavelength detection at 235 and 360 nm.

In this study, we report a validated HPLC method for the simultaneous measurement of vancomycin and CDP-1 in human serum. This method uses a small sample size (200 μl), solid phase extraction, a moderate HPLC gradient and single wavelength detection at 210 nm. The method has been used to verify the calibrator concentrations for the recently developed Vancomycin II (monoclonal) FPIA immunoassay and to assay clinical samples.

## 2. Experimental

### 2.1. Materials and reagents

Vancomycin HCl reference standard was obtained from US Pharmacopeial Convention, Rockville, MD. Cefazolin, sodium salt, was purchased from Sigma, St. Louis. CDP-1 (99 + %) was synthesized in house as a mixture of the major (-M) and minor (-m) isomers.

Acetonitrile and methanol were HPLC grade. Mono- and di-basic potassium phosphate were reagent grade. Sep-Pak Vac 3cc C18 extraction cartridges, PN20805 (500 mg with 0.8 ml hold-up volume) were ordered from Waters Chromatography Division of Millipore, Milford, MA.

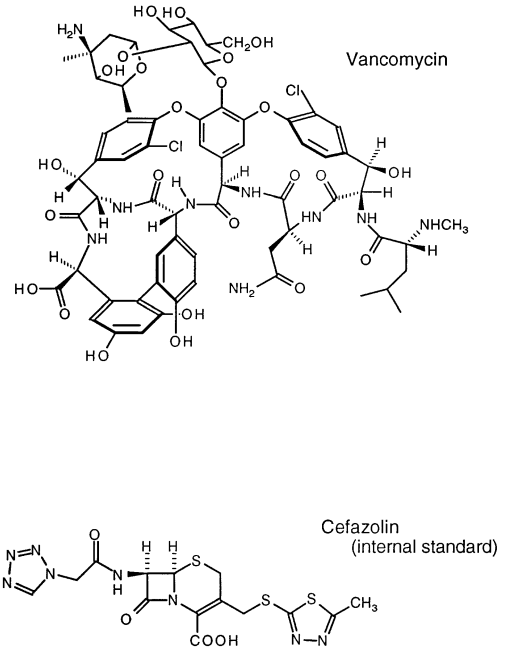


Fig. 1. Structures of vancomycin and cefazolin (internal standard).

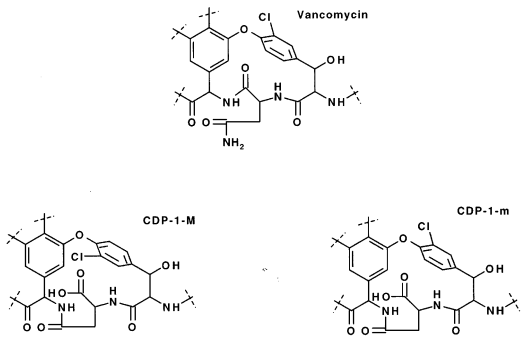


Fig. 2. Rearrangement of vancomycin to the degradation products CDP-1-M and CDP-1-m.

### 2.2. Standards

Stock vancomycin solution was prepared according to USP instructions by diluting the entire vial contents with distilled water to a concentration of 1 mg $ml^{-1}$. Working standards were made by dilution in human serum for a range of 1–100 μg $ml^{-1}$. Standard concentrations were 1, 2.5, 5, 10, 20, 25, 50, 60, 80 and 100 μg $ml^{-1}$. Controls were prepared at concentrations of 7, 35 and 70 μg $ml^{-1}$. Standards and controls were aliquotted to 2 ml vials and frozen until use.

CDP-1 was dissolved in 1 M sodium bicarbonate to make a 1 mg $ml^{-1}$ stock. Working standards in human serum were made with a concentration range of 1–25 μg $ml^{-1}$. Standard concentrations were 1, 2.5, 5, 10 and 25 μg $ml^{-1}$. Controls were prepared at concentrations of 7 and 20 μg $ml^{-1}$. Standards and controls were aliquotted to 2 ml vials, refrigerated overnight to allow isomer equilibration, then frozen until use.

Cefazolin was dissolved in distilled water to make a 1 mg $ml^{-1}$ stock. It was diluted with water to the working standard concentration of 100 μg $ml^{-1}$.

### 2.3. Equipment and instrumentation

Solid phase extractions were performed using a 12-port vacuum manifold (Alltech, Deerfield, IL). Excess organic solvent was evaporated by an N-Evap, Model 112 (Organomation, Berlin, MA). Extracts were centrifuged with an IEC Clinical Centrifuge (IEC, Needham Heights, MA).

Analysis by HPLC was performed using a Waters Model 600E pump, with a Waters Model 994 photodiode array detector. Injections were made by a Waters Model 710B wisp autoinjector (Waters, Milford, MA). Integrations were performed by a Hewlett Packard HP3394A integrator (Hewlett Packard, Palo Alto, CA). The HPLC was equipped with a YMC Pack ODS-AQ column, $250 \times 4.6$ mm I.D., Particle S-5 μm, 120A, preceded by guard column KGCQ-324C (YMC, Wilmington, NC).

### 2.4. Chromatography

Buffer was made by dissolving 11.94 g $KH_2PO_4$ and 2.14 g $K_2HPO_4$ in 2000 ml distilled water (pH 6, 0.05 M Phosphate) [20]. Mobile phase components A and B consisted of buffer–acetonitrile–methanol (91:5:4, v/v/v) and buffer–acetonitrile–methanol (84:8:8, v/v/v) respectively. Separations were accomplished using the following gradient conditions at a flow rate of 1.5 ml/min: 100% A for 2 min, to 100% B in 9 min, hold 14 min, to 100% A in 5 min and a hold for 5 min. The total run time for 1 sample was 35 min.

### 2.5. Sample preparation

Extraction columns were conditioned by washing with 2 column volumes of methanol, followed by 1 column volume of water, using enough vacuum to maintain a moderately steady drip. To achieve linearity over the wide range of vancomycin FPIA calibrators and sensitivity at the lower concentrations of CDP-1, separate runs of samples and standards were made from 1 to 25 μg $ml^{-1}$ (low curve) and from 25 to 100 μg $ml^{-1}$ (high curve). Selection of the low or high curve for HPLC analysis of vancomycin in the patient samples was based on the predetermined FPIA values.

In a small culture tube, 200 μl of distilled water was added to 200 μl of standard or sample followed by 50 μl of internal standard for the low curve or 200 μl of internal standard for the high curve. The tube was vortexed to mix and the contents were transferred to the extraction

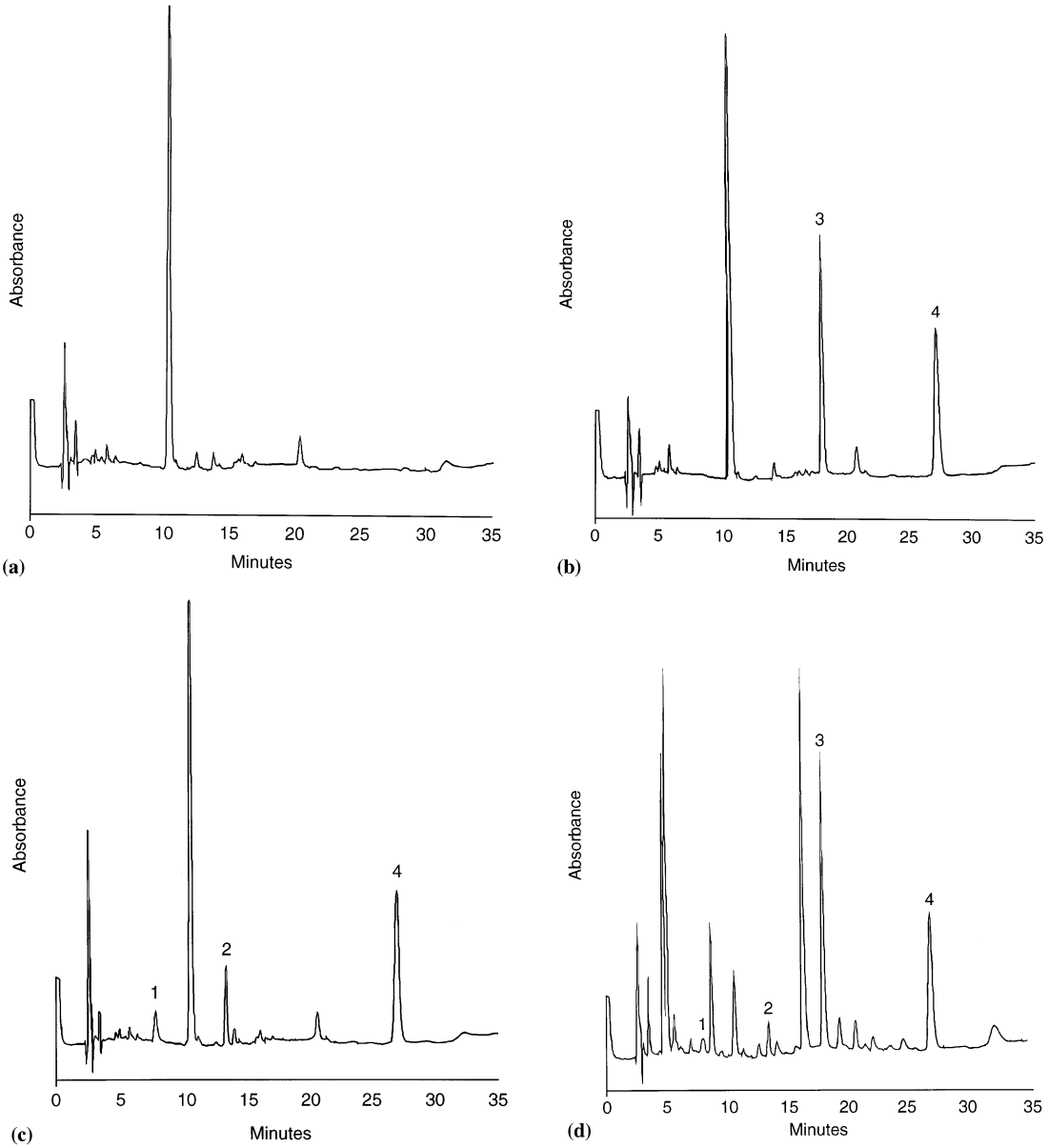


Fig. 3. Representative chromatograms of: (a) drug free human serum; (b) spiked serum containing 10 μg $ml^{-1}$ vancomycin; (c) spiked serum containing 2.5 μg $ml^{-1}$ CDP-1; and (d) a patient's serum containing 12.0 μg $ml^{-1}$ vancomycin and 2.5 μg $ml^{-1}$ CDP-1. Peaks: 1, CDP-1-m; 2, CDP-1-M; 3, vancomycin; 4, cefazolin (internal standard).

columns. The samples were aspirated into the columns and the columns washed with 2 column volumes of water. The samples were eluted with $2 \times 400$ μl of 0.05 M $KH_2PO_4$-acetonitrile (30:70, v/v) [22], followed by $2 \times 400$ μl of acetonitrile-water (50:50, v/v). The eluates were placed in the N-Evap at 50°C under a 10 $l/min^{-1}$ nitrogen flow for 10 min to reduce the organic content to a concentration more compatible with the mobile phases.

Table 1
Statistical data for vancomycin and CDP-1 calibration curves

| Concentration range (μg ml$^{-1}$) | Correlation coefficient | Slope | Intercept |
|---|---|---|---|
| (a) Vancomycin | | | |
| 1–25 | $0.999 \pm 1.17 \times 10^{-3}$ | $0.109 \pm 1.60 \times .10^{-2}$ | $0.0591 \pm 1.27 \times 10^{-1}$ |
| 25–100 | $0.997 \pm 2.03 \times 10^{-3}$ | $0.0313 \pm 6.66 \times 3^{-3}$ | $0.0270 \pm 4.49 \times 10^{-2}$ |
| (b) CDP-1 | | | |
| 1–25 | $1.000 \pm 1.65 \times 10^{-3}$ | $0.0493 \pm 5.07 \times 10^{-3}$ | $0.0139 \pm 1.31 \times 10^{-2}$ |

Eluates were cooled and centrifuged for 4 min at the highest speed setting ($\approx$1450–1475 g). Aliquots of the supernatant were transferred to injector vials and 50 μl was injected for the low curve or 25 μl for the high curve.

## 3. Results

The complete structures of vancomycin and cefazolin (internal standard) are shown in Fig. 1. Fig. 2 illustrates the rearrangement of vancomycin to CDP-1-M and CDP-1-m.

Representative chromatograms of extracts of blank serum, serum spiked with vancomycin and CDP-1 and serum from a renally impaired patient are shown in Fig. 3.

Quantitation was based on the peak area ratios of vancomycin or CDP-1-M to the internal standard (cefazolin) referenced to the respective standard curve provided with each analytical run.

The high curve for vancomycin was linear from 25 to 100 μg ml$^{-1}$. The low curves for vancomycin and CDP-1 were linear from 1 to 25 μg ml$^{-1}$. CDP-1 standards were made in the low range only.

Statistical data reported in Table 1 represent the average correlation coefficient, slope and intercept for 4 separate standard curves in each range. Each point on the standard curve is an average of 2 determinations. Limit of quantitation was 1 μg ml$^{-1}$.

The accuracy of the method was tested by preparing 2 independent sets of standards and controls in serum. The 2 sets were analyzed by 2 different analysts on different days. Accuracy was determined by the mean ($n = 6$) percent deviation for each level of the second set from the target concentrations. A detailed accuracy study is presented in Table 2.

Table 3 presents precision data at the control levels for vancomycin and CDP-1. Vancomycin precision was determined by running controls in replicates of 5 on 2 different days by 2 different analysts. CDP-1 controls were run in replicates of 4 on 2 different days by 2 different analysts.

## 4. Discussion

The stationary phase of the YMC-Pack ODS-AQ column included a monomeric bonding of C18 ligand (16% carbon) to a silica support with the addition of a hydrophilic endcap [27]. In this study, the ODS-AQ column gave consistently good separations (Fig. 3) that were not possible with conventional C18 columns. Additionally, the column exhibited no evidence of hydrolysis when operated under the mostly aqueous elution conditions. Although peaks of interest were well separated from serum peaks, a certain amount of care must be taken in mobile phase preparation. Slight changes in the organic solvent (methanol/acetonitrile) content of the mobile phase altered the relative position of the peaks of interest.

Interferences by theophylline, acetaminophen and salicylates were noted in some HPLC methods [21]. An advantage in the present method is that acetaminophen and theophylline, while extracted, were separated from vancomycin and CDP-1. Salicylates were poorly extracted and also well separated.

The use of ristocetin as the internal standard was reported [12,18,20,21] but in this method it

Table 2
Accuracy of vancomycin determination across the assay range

| Concentration (target) μg ml$^{-1}$ | Mean ($n = 6$) conc. (determined) μg ml$^{-1}$ | S.D. | R.S.D. % | Accuracy % |
|---|---|---|---|---|
| Calibrators | | | | |
| 5.0 | 4.83 | 0.22 | 4.4 | −3.4 |
| 10.0 | 8.94 | 0.34 | 3.8 | −10.6 |
| 25.0 | 26.06 | 2.55 | 9.8 | +4.2 |
| 50.0 | 48.00 | 2.48 | 5.2 | −4.0 |
| 100.0 | 103.00 | 5.29 | 5.1 | +3.0 |
| Controls | | | | |
| 7.0 | 6.54 | 0.34 | 5.2 | −6.6 |
| 35.0 | 37.37 | 1.19 | 3.2 | +6.7 |
| 70.0 | 71.03 | 2.49 | 3.5 | +1.4 |

Table 3
Precision data

| Concentration (target) μg ml$^{-1}$ | Mean ($n = 10$) conc. (determined) μg ml$^{-1}$ | S.D. | R.S.D. % |
|---|---|---|---|
| (a) Precision data on vancomycin controls | | | |
| 7.0 | 7.14 | 0.36 | 5.0 |
| 35.0 | 33.57 | 2.89 | 8.6 |
| 70.0 | 64.10 | 2.11 | 3.3 |
| (b) Precision data on CDP-1 controls (major isotope) | | | |
| 7.0 | 7.14[a] | 0.37 | 5.2 |
| 20.0 | 20.75[a] | 0.58 | 2.8 |

[a] Mean ($n = 8$).

eluted as a broad, tailing peak near CDP-1-M. Cefazolin [22] was chosen because it elutes later in an area with minimal interferences.

Initially, separate standards were made for vancomycin and CDP-1 so that any deterioration of vancomycin could be detected. No conversion of vancomycin to CDP-1 was noted during extraction and chromatography, therefore, standards containing both analytes could be used.

In 83 uremic patient samples analyzed by this method, the vancomycin concentrations ranged from 3.1 to 56.7 μg ml$^{-1}$. Seventy-six of these samples contained CDP-1 at concentrations of 1–10.2 μg ml$^{-1}$. A single standard curve with a range of 1–50 μg ml$^{-1}$ could be used for most patient samples. Additionally, this method was used to analyze 200 clinical samples for a correlation study during the development of the Vancomycin II (monoclonal) assay for the Abbott AxSYM™ Immunoassay Analyzer [28].

In conclusion, this HPLC method offers a simple, reliable assay for the simultaneous measurement of vancomycin and the major and minor isomers of its degradation product, CDP-1, in the serum of patients suffering renal impairment.

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