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Analytical Techniques for Vancomycin - A Review

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Abstract Vancomycin belongs to the vancomycin-ristocetin family of glycopeptides, and is a subclass of linear sugar containing peptides composed of seven amino acids. Its stereochemical configuration forms the basis of a particular mode of action, though its complexation with the D-alanyl-D-alanine terminus of peptidoglycon monomer. The glycosylated hexapeptide chain consists of chloro-β-hydroxytyrosines, p-hydroxyphenylglycines, N-methylleucine and aspartic acid forms a rigid molecular frame work and gives the difficulty in the analysis Vancomycin in the serum samples is usually estimated by liquid chromatography and the bacterial sensitivity was genereally tested by the microbiological assay. The present review deals with the qualitative, quantitative, microbiological and immunological assays and the comparison of the quantitative methods. Clinical implications of vancomycin have also been cited in the review.

Keywords: vancomycin, dalbaheptapeptides, *amylocolaptosis orientalis*, glycopeptide, analytical methods

INTRODUCTION

Vancomycin is biosynthesized by *Amylocolaptosis orientalis (Nocardia orientalis)* belonging to the family *Nocardiaceae*, which was first isolated from soil samples of Indonesia and India. Vancomycin belongs to the family of glycopeptide antibiotics. Its mode of action is inhibition of cell wall synthesis of susceptible bacteria. The main target of this antibiotic is the (L-Lys)-D-alanyl-D-alanine terminal peptide of the cell wall precursor. The interaction prevents the precursor from being added to the growing cell wall. In addition vancomycin alters the bacterial cell membrane permeability and RNA synthesis. Five of the seven amino acids that form the peptide skeleton are common to all dalbaheptides: Structurally, vancomycin has a glycosylated hexapeptide chain rich in amino acids, choloro-β-hydroxytyrosines, p-hydroxyphenylglycines, N-methylileucine and aspartic acid. Many of dalbaheptides contain aromatic rings that are cross-linked by aryl ether bonds into a rigid molecular frame work. Vancomycin (Fig. 1) [1] consists of a facultatively β-hydroxylated tyrosine, two p-hydroxyphenylglycine, a β-hydroxytyrosine and a m,m`-dihydroxyphenylglycine at carboxy terminal. Ether bonds between the phenyl moieties of amino acids facultative β-hydroxylated tyrosine, p-hydroxyphenyl glycine and β-hydroxytyrosine form adjacent rings. Another ring is formed by carbon-carbon bond between the phenyl moieties of amino acids *p*-hydroxyphenyl glycine and m,m`-dihydroxyphenylglycine at carboxy terminal. The remaining two amino acids help to classify all known dalbaheptides into four types. Vancomycin has aliphatic amino acids, usually leucine and asparagine. Dalbaheptides of natural origin have small variations in the nature and positions of substituents. Additional chemical features are chlorine atoms (upto four), methyl and additional hydroxyl groups that can be present in different positions in the

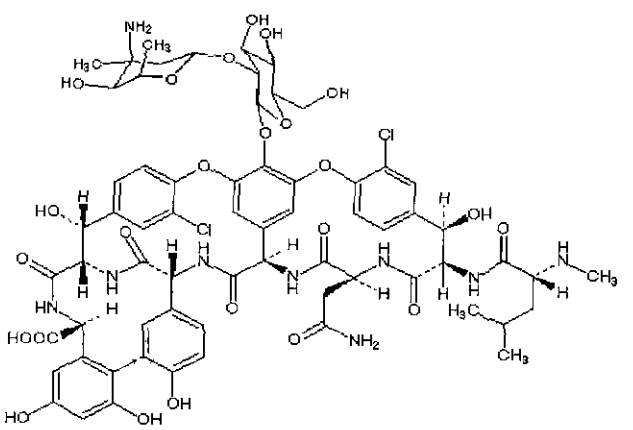

Fig. 1. Structure of vancomycin.

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Table 1. Compilation of solvents and conditions for vancomycin assay by thin layer chromatography

Stationary phase	Mobile phase	Developing temperature (°C)	Rf value	Time (h)
Reverse phase	5% Ammonium hydrogen carbonate:di-oxane (3:7)	4	0.37	6
Silica gel 60W	1% Ammonium hydrogen carbonate. Methanol (9:1)	20	0.46	2
Silica gel 60	1% Ammonium hydrogen carbonate: Propane-2-ol (9:1)	20	0.86	6
CMC	0.9% Diammonium ortho-phosphate, pH 8.3	28	0.52	6

phenyl residues. Vancosamine, epi-vancosamine, ristosamine, actinosamine and acosamine were the first isolated sugars from dalbaheptides. Many of these antibiotics are groups of strictly related factors called complexes. Among them, vancomycin and teicoplanin are used clinically as a result of high activity against gram positive pathogens such as many coagulase negative *Staphylococcus*(CNS), *Corynebacterium*, *Clostridium difficile*, multi-resistant *Staphylococcus aureus* and gentamicin resistant *Enterococcus* which are refractory to established drugs. Eremomycin is under clinical evaluation.

QUANTITATIVE AND QUALITATIVE ASSAYS OF VANCOMYCIN

Chromatographic Assay of Vancomycin

Thin layer chromatography (TLC): TLC systems have not very widely used in analysis of vancomycin, compared to other chromatographic separations. Thomas and Newland [10] have reported four TLC systems. These have been compiled in Table 1. The plates are over a distance of 150 mm, air dried and sprayed with freshly prepared aqueous solution of p-nitrobenzenediazonium tetrafluoroborate (1 mg/mL).

Paper chromatography: An official 1985 US Pharmacoepia [2] describes descending paper chromatography· 5 μL of vancomycin from a stock of 1.3 mg/mL is spotted on a Whatman No. 1 filter paper and runned for 7 h on butyl alcohol.water:pyridine (6:4:3), the paper is then dried and cut to accommodate on a lawn of standard bacterial culture. The data has been compiled in Table 2. The plate is incubated for 18 h at 37°C

Liquid chromatography: Most of the literature deals with the estimation and assaying of vancomycin in serum samples, with a couple from fermented broths. Due to the complex nature of blood serum, samples may have to be processed to remove most of the blood

Table 2. Solvent systems recommended for paper chromatography for vancomycin assay

Filter paper	Solvent system
Whatman No.4	Aqueous 80% ethanol v/v and sodium chloride buffer with 0.95 M sodium sulfate and 0.05 M sodium hydrogen sulfate monohydrate.
Whatman No.1	N-butanol:acetic acid:water (2:1:1).

Table 3. Compilation of the general methodology for vancomycin assay

Sample preparation and separation	Reference
Ion exchange followed by RP C_{18} separation	11
Liquid-liquid extraction followed by RP C_{18} separation	12
Liquid-liquid extraction followed by RP C_{18} separation using paired ion mobile phase	14
Protein precipitation and separation using CN HPLC columns	13
Bond elute [C_8] solid phase extraction and RP C_{18} separation	22

proteins as they could interfere with assay. General methods of sample preparation are given in Table 3.

Typical examples of solvents for precipitation of serum proteins include acetonitrile [13], cold trichloroacetic acid [14], sodium octanesulfonate and disodium EDTA [15] and isopropanol-acetonitrile [16]. It was reported that injection of untreated human serum into silica columns leads to rapid column deterioration, as the eluents utilised are almost totally aqueous and consequently prevents precipitation of serum proteins on the column.

Extensive efficiences with Sephadex columns is between 60-70% for both vancomycin and ristocetin (internal satandards). However acetaminophen and theophyllin interferes at therapeutic concentrations. The standard curve is linear to at least 100 mg/L with the minimum detectable limit of 3 mg/L. For control serum samples of 20 and 60 mg/L, between run precision is 8.6 and 11.5% respectively, and within-run precision is 3.1 and 4.2% respectively. Sztaricskari *et al.* [17] have separated seven antibiotics of the vancomycin type. By application of Lichrosorb RP-8 column and using 12% methylcellulosolve in sodium citrate buffer (pH 6.4) as eluent, they have reported separation of ristomycin. A major component of A-35512 B antibiotic complex and from avoparicin X and B. A simple quantitation of vancomycin by HPLC has reported by McClair *et al.*, [14] where the total analysing time is 20 minutes. This method does require internal standards, but neither sample extractions nor sample derivitazation. The stan-

Table 4. Compilation of columns, mobile phases and detection wave lengths for vancomycin assay by HPLC

Column	Mobile phase	Detection (nm)	Reference
RP Micro bondapak C_{18}	12% Acetonitrile+88% 0.01 M 1-heptane sulphonic acid	280	16
Aminopropyl shimpak CLC NH2	0 07 M phosphate buffer, pH 2.5:Acetonitrile (30·70)	240	15
ODS (3μ)	0 02M ammonium acetate: Acetonitrile (91·9)	214	20
Micro bondapak C_{18}	Methanol: 0 1 M diammonium hydrogen phosphate pH 6 (26 74)	2 54	21
Supelco LC18	(Sodium octane sulfonate + Disodium EDTA) + Acetonitrile+Methanol	230	17
Nucleosil C18	5mM potassium dihydrogen phosphate (pH 2.8) + 22% Methanol	229	14
RP Supelcosil RP C_{18}	0.05 potassium dihydrogen phosphate (pH 5.0): Acetonitrile (90.10)	254	22
PLRP-S 100A	8% Acetonitrile in 0.02M borate buffer, pH 3.2	235	10
ODS	Acetonitrile.Tetrahydrofuran· triethyl amine buffer pH 3.2 (29:1:70)	280	23
RP ODS C18	Acetonitrile in 0.05 sodium hydrogen phosphate pH 6.0	235/360	18

Table 5. List of internal standards used for HPLC analysis of vancomycin

Internal standards	Reference
Vitamin B12	18, 21, 24
Cefazolin	22
Trimethoprim	14
3,4,5 trimethoxy phenyl acetonitrile	17

dard deviation of the assay at 5 μg/mL is ± 0.25 μg (Table 4). A variety of internal standards have been used for the assaying of vancomycin by HPLC, which is compiled in Table 5. A compilation of the drugs that do not interfere in the HPLC analysis is shown in Table 6

Microbiological Assay of Vancomycin

A standardized disk method has been recommended by the National Committee for Clinical Laboratory Standards (NCCLS) [23,24] to test the susceptibility of vancomycin. Susceptibility test with a 30 μg vancomycin hydrochloride disk should be interpreted as given in

Table 6 List of drugs found not to interfere with the vancomycin by HPLC [16]

Antibiotics	Antineoplastics	Other drugs
Amikacin	Adriamycin	Acetaminophen
Amphotericin B	Allopurinal	Acetazolamide
Benzyl penicillin	Cisplatin	Aspirin
Carbenicillin	Cyclophosphamide	Carbamazepine
Cefoxitin	Cytarabine	Chlorpromazine
Cefuroxime	Dactinomycin	Diazepam
Chloramphenicol	5-Fluorouracil	Ethosuximide
5-Flurosytosine	Methotrexate	Furosemide
Gentamycin	Thioguanine	Phenobarbital
Kenamycin		Phenytoin
Ketooconazole		Primidone
Miconazole		Procainamide
Penicillin G		Quinidine
Tobramycin		Theophylline

Table 7. Interpretation of standard test results for vancomycin assay [3]

Disk test zone diameter	Target organism susceptibility	Dilution method (MIC) (μg/mL)
> 12	Highly susceptible	< 4
10-11	Intermediate susceptible	4 – 16
< 9	Resistant	> 16

Table 8. List of minimum inhibitory concentrations (MIC's) for some selected organisms

Selected organism	Minimum inhibitory concentration (MIC) (μg/mL)
Staphylococcus aureus TOUR	0.25
Staphylococcus epidermidis ATCC 12228	0.5
Enterococcus fecalis ATCC 7080	0.5
Streptococcus pyogenes C 203	0.13
Clostridium perfringes ISS 30543	0 13
Neisseria gonorroheae ISM 68/126	32.0
Staphylococcus epidermidis ATCC 29213	0.5-2.0

Table 7.

Organisms in the intermediate susceptibility range are likely to respond to vancomycin treatment if infection is confined to tissues or fluids in which high antibiotic concentrations are allowed. Minimum inhibition concentration (MIC) of some of the selected microorganisms are given in the Table 8.

The last two organisms given in Table 8, *Staphylococcus aureus* ATCC 29213 and *Enterococcus fecalis* ATCC 29212, are the microorganisms recommended by the NCCLS [23] for the standard dilution method. For the disk test, 30 μg of vancomycin should give a zone diameter between 15-19 mm for *Staphylococcus aureus* ATCC 25923 [23]. For a 30 μg vancomycin disk how-

ever, Barry *et al.* [24] reported a zone smaller than recommend 10 mm for resistant and larger than 15 mm for sensitive organisms category.

Immunological Assays: One of the commonly used immunological assays is the FPIA (Fluorescence polarization immunoassay) This is based on the changes in fluorescence polarization by binding with antibody [28]. In one such study, Schwenzer *et al.* [26] have extended this concept to the detection of vancomycin Fluorescein-labelled vancomycin is employed as a tracer along with the antisera for vancomycin, which has been raised in rabbit for conventional immunological techniques. The tracer, sample and dilute antiserum are combined and polarization of tracer fluorescence is detected in the fluorometer. This measurement is free of interference from hemolysis, bilirubin and protein concentration changes. This assay is also reported to be free of matrix effects and no interference is reported in spiked hemolytic, lipemia or icteric sera [26]. This assay compares well with microbial and HPLC assays. Repetitive analysis of controls containing 73.5 and 75 μg of vancomycin by FPIA methods yields a coefficient of variation of 3.0 and 2.3 respectively [26].

A commercial diagnostic kit based on FPIA technology, is now available under the trade name, TDx, by Abbott Labs, Diagnostic Division, North Chicago, IL, USA [28]. A study was carried out by Joos *et al.* [28], to detect the accuracy of the Abbott TDx FPIA kit over a period of six years. A comparison of 209 assay results with externally supplied target concentrations showed a good correlation without any deviations from linearity. They further report that on an average, the same calibration curves could be used over a period of 19 weeks. Jandreski and Garbincius [37] reported that the sensitivity of the Abbot TDx system was four-fold in cerebrospinal fluid as compared to serum thereby matching the cerebrospinal fluid as an acceptable specimen for this system.

Other Qualitative Analytical Methods for Vancomycin: Complexation of vancomycin with Cu^{2+} has been used for the detection of vancomycin by a continuous flow method and amperometric detection in a polarographic cell of the thin layer type.

The mode of action of this family of antibiotics, that is binding to the D-alanyl-D-alanine terminus has been exploited to detect vancomycin. SPERA (Solid-phase enzyme receptor assay) is one of such assay systems that utilizes the mode of action of the antibiotic binding of D-alanyl-D-alanine [30]. Synthetic analogues of the biological receptor albumin-ε-aminocaproyl-D-alanyl-D-alanine achieved 50% displacement with vancomycin concentrations ranging from 0.04 to 4 mg/mL. Apparently, SPERA is claimed to work even in complex media. Activated CH-Sepharose on D-ala-D-ala for vancomycin and teichoplanin shows affinity constants to be around 10^5 L/mol and the effective binding sites to be 6-7 μEq/mL gel.

Table 9. Comparison of correlation coefficients against various assay methods for vancomycin

Assay methods compared	Correlation coefficient
FPIA Vs HPLC	0 97 [28], 0.9996 [34], 0.967 [12]
FPIA Vs Microbial assay	0.90 [28], 0.985 [33], 0.7773 [34]
HPLC Vs Microbial assay	0.90 [28], 0.7779 [34]
HPLC Vs RIA	0 945 [13]

Comparison of Quantitative Methods

A number of groups have reported a comparison between the various assay methods. Table 9 compiles the correlation coefficients of the various methods reported. A laboratory evaluation of the five methods (Bioassay, HPLC, FPIA, FIA (Fluorescence immunoassay) and RIA (Radioimmunoassay)) has been reported [33]. They reported that FPIA is the most precise and FIA is the least accurate. Intra-run coefficients of variation of FPIA were 0.9 to 3% as compared 8.9 to 14.5% for FIA. Inter-run coefficient of variation was 2.8 to 8.1% for FPIA as compared to 12.2 to 16.2% for FIA. RIA is reported as inconvenient as it requires an extra dilution of the specimen and an additional 64 μg/mL standard for specimens having 32-64 μg vancomycin/mL. Based on the rapid turn around time and stability of the standard curve, FPIA is the best. Pohlod *et al.*, [30] in a comparison between FPIA and the microbial assay, reported that bioassay is the least expensive, though, more labour intensive. Ackerman *et al.*, [27] compared 123 samples from 34 patients treated with vancomycin using FPIA (Abbott, TDx) and standard RIA (American Diagnostic Corporation, CA, USA) diagnostic kits and found them comparable. During the pharmacokinetic studies of patients on continuous ambulatory peritonial dialysis (CAPD) treated with vancomycin, a discrepancy was noted when serum vancomycin concentration were assayed by HPLC and FPIA [34]. Peak serum concentration by HPLC showed 36.3, 32.2 and 31.6 μg/mL as compared to 42.1, 43.1 and 45.6 μg/mL by FPIA, in *in vitro* study of vancomycin in serum samples. The results showed a degradation half-life of 693 h by FPIA method as compared to 210 h by HPLC. The authors reported that in the CAPD patients, vancomycin degradation products accumulated leads to an overestimation of vancomycin concentrations in sera when measured by FPIA. This particular aspect has been studied in detail by White *et al.* [34]. Vancomycin stored in serum, phosphate-buffered saline and CAPD fluid at 37°C for 10 days when assayed by HPLC, microbial assay and FPIA (TDx assay) gave some interesting results. While HPLC and microbial assay agreed well and indicated about 50% loss over 10 days in serum and phosphate-buffered saline, FPIA showed losses of 20% and 40% respectively. The antibiotic degradation products were purified by HPLC and were found to cross-react with the TDx assay. This suggests that the TDx assay become non-specific in presence of vancomycin degrada-

Table 10. Organisms sensitive to vancomycin

Genus	Species
Staphylococci	*aureus*
Staphylococci	*epidermidis*
Streptococcus	*pyogenes*
Streptococcus	*pneumoniae*
Streptococcus	*agalactiae*
Streptococcus	*bovis*
Enterococcus	*fecalis*
Clostridium	*difficile*

tion products, thereby overestimating the actual antibiotic concentration. Since FPIA technology utilizes a polyclonal antibody system, another system called EMIT has been developed for vancomycin assay which utilizes monoclonal antibodies specific for vancomycin [16]. While EMIT/FPIA system is recommended by Hu *et al.* [18] for monitoring the antibiotic concentrations in renally impaired patients, problems have been reported by Yeo *et al.* [35] for the EMIT system. During a clinical performance evaluation of the EMIT and FPIA vancomycin assays, it was found that, though acceptable at concentrations less than 30 mg/L by EMIT system the precision was marginal in the range greater than 30 mg/L. Further on comparison with FPIA, EMIT shows the following correlation:

$$[\text{EMIT}] = (0.877)\,(\text{TDx}) + 0.435\ \text{mg/L}$$

This proportional bias has been reported to be more pronounced in specimens with high levels of creatinine as compared to normal levels of creatinine, thereby recommending the EMIT vancomycin be used with caution [36].

CLINICAL IMPLICATIONS OF VANCOMYCIN

Bactericidal action results primarily from inhibition of cell wall biosynthesis. In addition, vancomycin alters the bacterial cell membrane permeability and RNA synthesis. There is no cross resistance between vancomycin and other antibiotics. Organisms sensitive to vancomycin have been compiled in Table 10.

Vancomycin is not active in-vitro against Gram negative bacilli, mycobacteri and fungi.

Vancomycin is indicated for full treatment (or initial therapy) of serious or severe infections caused by susceptable strains of methicillin resistant (β-lactam resistant) *Staphylococci* (MRSA). It is indicated for penicillin allergic patients, especially those who cannot receive or have failed to respond to other drugs including penicillins or cephalosporins.

Vancomycin is poorly absorbed after oral administration Injection is painful. Vancomycin is approximately 55% serum concentrations in 10 to 100 μg/mL. Vancomycin is excreted in human milk. In the first 24 h, about 75% of administered dose is excreted in urine. The mean elimination of half-life of the molecule from plasma is 4 to 6 h. The median lethal intravenous dose is 319 mg/Kg in rats and 400 mg/Kg in mice.

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