ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1981, p. 139-143
0066-4804/81/010139-05$02.00/0

Vol. 19, No. 1

Sensitive Radioimmunoassay for Vancomycin

KEI-LAI L. FONG,† DAH-HSI W. HO,[1]* LAURIE BOGERD,[1] THERESA PAN,[1] NITA S. BROWN,[1] LAYNE GENTRY,[2] AND GERALD P. BODEY, SR.[1]

Department of Developmental Therapeutics, The University of Texas System Cancer Center M. D. Anderson Hospital and Tumor Institute,[1] and Baylor College of Medicine,[2] Houston, Texas 77030

A radioimmunoassay for vancomycin has been developed which uses rabbit antiserum induced by vancomycin-bovine serum albumin conjugates and vancomycin labeled with ^{3}H or ^{125}I. Using either isotope, the method is simple and reproducible and has a sensitivity of 4 or 0.04 ng/ml, depending on the tracer used. This is 200- to 20,000-fold improvement in sensitivity compared with the most sensitive bioassay. Drug levels in serum or urine samples from patients receiving vancomycin can be determined by this assay procedure without processing. The data obtained with ^{3}H and ^{125}I labels were in good agreement. Patients' plasma vancomycin concentrations determined by radioimmunoassay correlated well with those determined by bioassay when the drug was administered intravenously. However, after oral administration the drug could be detected only by radioimmunoassay. The antiserum was evaluated for cross-reactivity with a wide variety of antibiotics and cancer chemotherapeutic agents, and no significant interference was found.

Vancomycin was isolated from *Streptomyces* sp. in 1956 (14) and was first introduced into clinical practice in 1958 (1). It is primarily active against gram-positive bacteria and affects cell wall synthesis by forming a complex with a peptide precursor (9). The chemical components of the antibiotic have been studied since 1964 (13, 17, 18, 22), and the complete molecular structure was determined by X-ray analysis in 1978 (16). Clinically, vancomycin is used mainly for severe staphylococcal infections, especially when the responsible strain is resistant to the more commonly used antibiotics or the patient is allergic to penicillins and cephalosporins (5). It has also been included in oral prophylactic regimens for cancer patients (3). Vancomycin is associated with nephrotoxicity and ototoxicity (5), and serum concentrations of 80 to 100 μg/ml have been associated with serious toxic effects (11). The distribution of vancomycin in plasma, urine, and tissue has been studied previously by microbiological assays (1, 7, 8, 19). This paper describes a sensitive radioimmunoassay developed for vancomycin. The assay provides a rapid and simple procedure for quantitating vancomycin and therefore should be more suitable for monitoring plasma vancomycin concentrations as well as for studying the pharmacology of the antibiotic.

MATERIALS AND METHODS

Vancomycin hydrochloride was obtained from Eli Lilly & Co., Indianapolis, Ind. *N*-Succinimidyl[2,3-^{3}H] propionate (specific activity, 43 Ci/mmol), $Na^{125}I$ (specific activity, 13 to 17 mCi/μg), and Phase Combining Solvent were purchased from Amersham Corp., Arlington Heights, Ill. Dextran T70 was from Pharmacia, Uppsala, Sweden. Norit A charcoal was obtained from Fisher Scientific, Fair Lawn, N.J. Bovine serum albumin (BSA), Freund complete adjuvant, and other chemicals are commercially available. Silica gel plates (Silica Gel F254) and carboxymethyl cellulose (CM52) with an exchange capacity of 1 meq/g (dry weight) were purchased from Whatman, Inc., Clifton, N.J. Female New Zealand rabbits, approximately 2.5 kg and 12 to 15 weeks of age, were purchased from Rich-Glo Co., El Campo, Tex.

Preparation of vancomycin-BSA conjugate. Vancomycin was conjugated to BSA by the water-soluble carbodiimide method. Vancomycin hydrochloride (252 mg) and BSA (50 mg) were dissolved in 15 ml of cold water, after which 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (320 mg) was slowly added to the solution with constant stirring. This reaction mixture was incubated for 3 h at room temperature and then overnight at 4°C. Cold acetone (24 ml) was added to precipitate the product, which was collected by centrifugation, washed twice with 5 ml of cold 60% acetone–water, and dried under vacuum (yield, 59 mg). Thin-layer chromatography (silica gel; solvent system, 1-butanol–1-propanol–2 N ammonium hydroxide [2:5:3]) of the conjugates showed a single spot at the origin as detected by ultraviolet light and iodine, indicating that the conjugate was free of vancomycin (vancomycin R_f, 0.45).

Immunization. Vancomycin-BSA conjugates suspended in 0.9% NaCl were emulsified with an equal volume of Freund complete adjuvant to obtain a concentration of 1 mg/ml. Three rabbits were immunized with 1 ml of the emulsion at multiple intradermal and subcutaneous sites. Booster injections (0.5 ml) were given at triweekly intervals. After 3 months of immu-

† Present address: Smith, Kline & French Research Laboratories, Philadelphia, PA 19101.

nization, heparinized blood was obtained from the rabbits' ear veins 1 week after each booster injection. Plasma was separated by centrifugation and stored in small portions at −70°C. The titer of the antiserum was determined by its binding ability to both ^{3}H- and ^{125}I-labeled vancomycin.

Another group of these rabbits was immunized with unconjugated vancomycin by the same procedure and schedule, except that each animal received 25 μg of vancomycin per injection.

Preparation of [^{3}H]propionylvancomycin. *N*-Succinimidyl[2,3-^{3}H]propionate (5 mCi in 25 ml of toluene) was evaporated to dryness under a stream of nitrogen. To this dried residue was then added 30 μl of vancomycin solution (5 mg/ml in 0.01 M phosphate buffer, pH 7.5), and the reaction mixture was incubated at room temperature for 2 h. The product was purified by column chromatography on a carboxymethyl cellulose column (0.6 by 20 cm). The unreacted succinimidylpropionate was eluted from the column with 0.01 M ammonium acetate, pH 6.0 (35 ml); the product was then eluted with 0.05 M ammonium acetate, pH 6.4 (10 ml), and the unreacted vancomycin was collected in later fractions with the same buffer. Because of the small quantity of the product, no attempt was made to estimate its specific activity.

Preparation of [^{125}I]iodovancomycin. Vancomycin was labeled with ^{125}I by using the chloramine-T method (15). To 1 mCi of $Na^{125}I$ in 20 μl of borate buffer (0.4 M, pH 7.5) were added 10 μl each of vancomycin (50 μg/ml) and chloramine-T (5 mg/ml). After 1 min of incubation at room temperature, the reaction was stopped by adding sodium metabisulfite (10 μl, 12 mg/ml) and potassium iodide (10 μl, 10 mg/ml). The mixture was then diluted with 200 μl of 0.01 M ammonium acetate, pH 6.0, and applied onto a carboxymethyl cellulose column (0.6 by 20 cm) packed in the same buffer. The unreacted ^{125}I was eluted with 0.01 M ammonium acetate. ^{125}I-vancomycin and vancomycin were eluted together with 0.05 M ammonium acetate, pH 6.5. The specific activity of the product was estimated to be 486 mCi/mg.

Radioimmunoassay by [2,3-^{3}H]propionylvancomycin. The assay was performed in 4-ml polypropylene tubes, each containing 0.1 ml of 0.01 M phosphate-buffered saline (0.01 M NaH_2PO_4–0.15 M NaCl containing 0.1% NaN_3), pH 7.5, 0.1 ml of antiserum (diluted 1:600 with 0.1% gelatin in 0.01 M phosphate-buffered saline), 0.1 ml of [^{3}H]propionylvancomycin with approximately 4,000 cpm, and an appropriate amount of vancomycin standard or unknown samples in a total volume of 0.5 ml. The binding reaction was initiated by adding antiserum and incubating the mixture at room temperature for 1 h. The tubes were then placed in an ice bath, and a 0.5-ml, ice-cold suspension of 2% activated charcoal and 0.1% dextran in phosphate-buffered saline was added. After incubation in ice for 15 min, the tubes were centrifuged in a PR-2 centrifuge (International Equipment Co.) at 900 × *g* for 15 min. The radioactivity in 0.7 ml of supernatant was counted in a Packard scintillation spectrometer after adding 10 ml of 66% Phase Combining Solvent in xylene. Data points for the standard curve and for unknown samples were determined in duplicate. The control binding was about 1,700 ± 50 cpm per tube and was corrected for the background (approximately 130 ± 20 cpm per tube) when antiserum was absent.

Radioimmunoassay by [^{125}I]iodovancomycin. For radioimmunoassay by [^{125}I]iodovancomycin, the same procedure was used as for [^{3}H]propionylvancomycin with the exceptions that approximately 20,000 cpm of [^{125}I]iodovancomycin was used in each assay tube and the antiserum dilution was 1:20,000. After 1 h of incubation at room temperature, the tubes were placed in ice, and 0.5 ml of a cold suspension of 2% charcoal and 0.1% dextran in phosphate-buffered saline was added. The tubes were further incubated in ice for 15 min and then centrifuged at 900 × *g* for 15 min. Supernatant (0.5 ml) was removed and measured for radioactivity in a Packard PRIAS gamma counter. The control binding was about 8,000 ± 400 cpm per tube, and the background was approximately 600 ± 50 cpm per tube.

Bioassay. Plasma vancomycin concentrations were also determined by the disk diffusion method, using *Bacillus subtilis* (ATCC 6633) as the test species. Details of this method were described previously by Bulger et al. (4). At various times heparinized blood was drawn from patients who had received 500 mg of vancomycin by a 30-min intravenous infusion or after 100 mg of the drug was administered orally in a 20-ml solution. Drug concentrations were determined by both radioimmunoassay and bioassay. Urine samples were also collected as voided from patients who had received intravenous vancomycin, and the drug content was determined by radioimmunoassay.

RESULTS

Antibody response. All the rabbits immunized with vancomycin-BSA conjugates produced antiserum against vancomycin; in 6 months they achieved a maximum titer of 1:100,000 with [^{125}I]iodovancomycin and 1:3,000 with [^{3}H]propionylvancomycin. However, the rabbits immunized for the same period of time with unmodified and unconjugated vancomycin did not produce any antibody at all, even though the molecular weight of vancomycin is 1,449 (20).

Sensitivity. The standard curve of vancomycin radioimmunoassay was linear on a logit-log plot (Fig. 1). In the ^{3}H-vancomycin assay, drug concentrations ranging from 4 to 100 ng/ml could be measured accurately, and this sensitivity could be increased 100-fold when ^{125}I-vancomycin was used (Fig. 1).

Effect of time on binding reaction. The effect of time on the binding reaction was studied at the 50% binding point. The binding of [^{3}H]-propionylvancomycin and the antiserum attained equilibrium very fast (about 15 min) and only increased slightly at longer time periods. For routine assay, an incubation time of 1 h was chosen for experimental convenience. In the case of the ^{125}I method, the binding reaction proceeded more slowly, probably due to the lower

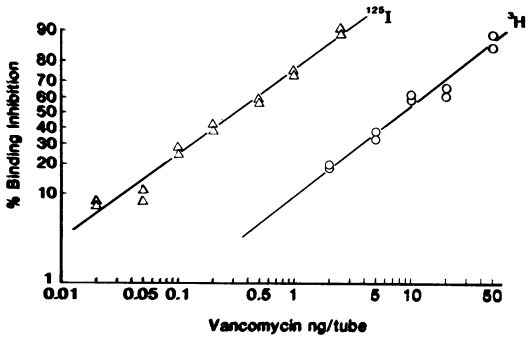

FIG. 1. *Standard curve of vancomycin radioimmunoassay with [^{3}H]propionylvancomycin and [^{125}I]iodovancomycin.*

concentrations of both [^{125}I]iodovancomycin and antibody in the reaction mixture. However, the standard curves performed at various incubation times (1, 2, 3, and 4 h) were very similar, and the standard deviation for 50% binding inhibition was <10%.

Assay accuracy and reproducibility. Plasma drug levels in patients receiving vancomycin were measured at random time points by the ^{125}I radioimmunoassay. The data obtained were similar on different days, with different operators, and with different amounts of plasma samples (Table 1). These plasma samples were also assayed with ^{3}H-vancomycin. Values from both methods were compared by linear regression analysis, and an excellent correlation (r^2 = 0.99) was obtained (Fig. 2). This suggests that the results obtained with either the ^{3}H or the ^{125}I tracer are in good agreement and further demonstrates the reproducibility of the assay.

Comparison of radioimmunoassay and bioassay. Plasma drug levels were determined by both methods after patients had received vancomycin (500 mg) by intravenous infusion. The correlation coefficients obtained by linear regression analysis were 0.98, 0.97, 0.87, and 0.86 for four different patient studies, an example which is shown in Fig. 3. Although it appeared that the values in Fig. 3 obtained by radioimmunoassay were lower than those by bioassay, the data in the other three patient studies showed the reverse. However, the results were not statistically different between radioimmunoassays and bioassays. In two patients the drug levels determined by radioimmunoassay did not correspond with those obtained by bioassay. Table 2 shows the results for one of the patients where the correlation coefficient between the two types of assays was only 0.31. These two patients were on amikacin and other aminoglycosides. *B. subtilis*, the species used for the bioassay, is also susceptible to aminoglycosides.

TABLE 1. *Patient plasma vancomycin concentrations determined by radioimmunoassay with ^{125}I tracer*[a]

Patient no.	Plasma vancomycin (μg/ml)[b]				
	Trial 1	Trial 2	Trial 3	Trial 4	Mean ± standard deviation
1	24.0	24.5	24.0	24.0	24.1 ± 0.3
2	9.6	8.1	8.2	9.2	8.8 ± 0.7
3	12.5	12.1	11.4	12.2	12.1 ± 0.5
4	8.1	9.4	9.0	9.2	8.9 ± 0.6
5	10.5	9.5	9.8	10.0	10.0 ± 0.4
6	64.0	57.5	56.0	48.0	56.0 ± 6.6
7	13.5	15.0	12.5	15.0	14.0 ± 1.2
8	10.0	10.0	10.2	11.0	10.3 ± 0.5

[a] Vancomycin was given by intravenous infusion. Dose and sampling time were not recorded.

[b] A plasma sample of 0.1 μl was used in the assay for trials 1 and 2, and a plasma sample of 0.05 μl was used in trials 3 and 4. Trials 1 and 2 were performed by two operators on the same day, trials 1 and 3 were performed by one operator on the same day, and trials 3 and 4 were performed by one operator on different days.

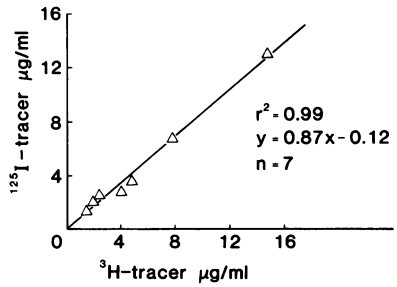

FIG. 2. *Correlation of plasma vancomycin concentrations as determined by radioimmunoassays with ^{125}I and ^{3}H labels.*

Thus, the erratic plasma levels obtained at various times could be attributed to the effects of other antibiotics. After an oral dose (100 mg in 20 ml), the vancomycin levels were measurable by radioimmunoassay. The plasma levels were 0.2, 0.3, 0.4, 2.2, and 8.3 ng/ml at 0.5, 1, 2, 3, and 4 h, respectively, after drug ingestion. However, no vancomycin could be detected in plasma as measured by bioassay in this 4-h period.

Urinary excretion. One patient received 500 mg of vancomycin intravenously over 0.5 h. At 4 h, 54% of the drug was excreted in urine. Another patient received 1,000 mg by 1-h intravenous infusion. At 2, 2.8, 3.7, and 4.3 h, the amounts excreted in the urine were 7.9, 13.8, 34.0, and 53.3%, respectively, and by 21 h the drug was essentially completely excreted.

Cross-reactivity. Since vancomycin is used

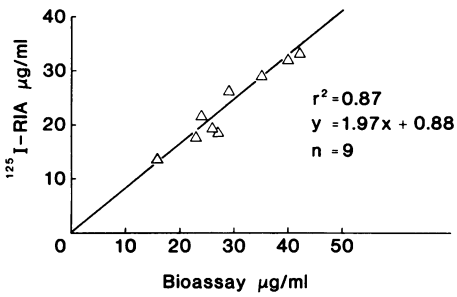

FIG. 3. *Comparison of plasma vancomycin concentrations as determined by ^{125}I radioimmunoassay (RIA) and bioassay.*

TABLE 2. *Effect of other antibiotics on bioassay of vancomycin*

Time	Vancomycin concn (μg/ml)	
	RIA[a]	Bioassay
0[b]	13.0 ± 1.5	ND[c]
0.25	6.7 ± 1.3	60
0.75	3.5 ± 0.4	72
1	2.7 ± 0.1	38
2	2.5 ± 0.2	48
4	1.8 ± 0.2	50
6	1.3 ± 0.3	42

[a]Results presented are means ± standard deviations from five determinations each (four with ^{125}I tracer and one with ^{3}H tracer).

[b]Immediately after the 30-min intravenous infusion of vancomycin (500 mg).

[c]ND, Not determined due to sample volume shortage.

in our hospital for treating infections in cancer patients, the assay was tested for interference by other antibiotics and cancer chemotherapeutic agents at a concentration of 2 μg/ml. Such drugs as amikacin, ticarcillin, tobramycin, rifampin, 5-fluorouracil, cytarabine, bruceantin, methotrexate, vincristine, vinblastine, doxorubicin, cyclophosphamide, and 4'-(9-acridinylamino)methanesulphon-*m*-anisidide showed no competition for binding to the antiserum with either ^{3}H- or ^{125}I-vancomycin. Since cyclophosphamide is activated in vivo, rat plasma containing cyclophosphamide and its metabolites was also tested for cross-reactivity. Thirty minutes after an intraperitoneal injection of cyclophosphamide at a dose of 500 mg/kg, rat plasma showed no effect on the competitive binding of the antiserum with ^{125}I-vancomycin.

DISCUSSION

The radioimmunoassay described here provides a rapid, sensitive, and precise method for quantitating vancomycin. With the high sensitivity of the assay, only a small amount of biological sample is required. The previous microbial methods could only measure vancomycin concentrations greater than 10 to 12 μg/ml (1), and no drug could be detected in rat or rabbit tissues after vancomycin administration (12). A recent report of a modified bioassay improves the sensitivity limit to 0.8 μg/ml (21), although it still has limitations in human tissue distribution studies (19). In addition, the test species used in the bioassay is also susceptible to other antibiotics, which can produce erratic data (see Table 2). Recently a radioimmunoassay for vancomycin with a sensitivity of 1 ng/ml became commercially available from Monitor Science Corp., Newport Beach, Calif. This assay yielded essentially identical results to those obtained by the microbiological method (6). Our present findings accord with these results. Two high-performance liquid chromatographic assays have also been developed for vancomycin (10, 20). The sensitivity of the high-performance liquid chromatographic methods is in the microgram-per-milliliter range, and samples had to be purified on an ion-exchange column before they could be subjected to high-performance liquid chromatography. Therefore, once the antiserum is produced, the present radioimmunoassay should provide a more convenient and sensitive method for investigating vancomycin pharmacology as well as for redefining the toxic levels in patients with normal and abnormal liver or renal function. Vancomycin has been chromatographically separated into three fractions with different biological potencies (2). Since vancomycin-BSA was conjugated with the complexed vancomycin, the antiserum produced would likely be cross-reactive with the different components in vancomycin preparations. High-performance liquid chromatography measures the major fraction of vancomycin (10, 20) and would be more specific than the radioimmunoassay reported here.

Both ^{3}H- and ^{125}I-vancomycin radioimmunoassays gave comparable and reproducible results. The ^{3}H label method, with a detection limit of 4 ng/ml, is sufficient for routine monitoring of plasma or urine drug levels when the antibiotic is given by intravenous infusion. However, the plasma level is very low when the drug is administered orally, and the sensitivity of the ^{125}I assay (100-fold higher than the tritium assay) is required for pharmacokinetics studies. Our preliminary studies also showed that the radioimmunoassay can be used for measuring drug levels in tissue samples. Therefore, complete pharmocological studies of vancomycin will be feasible with this newly developed radioimmunoassay. The cross-reactivities of the vancomycin antiserum with some commonly used anti-

biotics and cancer chemotherapeutic agents were tested. The drugs studied were of different molecular sizes and different structural characteristics, and none of them showed any interference with the vancomycin radioimmunoassay. This suggests that this assay is appropriate for clinical pharmacological studies even in patients who are also under other chemotherapy.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants CA-05831 and CA-11520 from the Division of Cancer Treatment of the National Cancer Institute.

LITERATURE CITED

1. **Anderson, R. S., M. M. Worth, P. N. Harris, and K. K. Chen.** 1957. Vancomycin, a new antibiotic. IV. Pharmacologic and toxicologic studies. Antibiot. Annu. **1956-1957**:75-81.
2. **Best, G. K., N. H. Best, and N. N. Durham.** 1969. Chromatographic separation of the vancomycin complex, p. 115-119. Antimicrob. Agents Chemother. 1968.
3. **Bodey, G. P., and B. Rosenbaum.** 1974. Effect of prophylactic measures on the microbial flora of patients in protected environment units. Medicine (Baltimore) **53**: 209-228.
4. **Bulger, R. J., D. D. Lindholm, J. S. Murray, and W. M. M. Kirby.** 1964. Effect of uremia on methicillin and oxacillin blood level. J. Am. Med. Assoc. **187**:319-322.
5. **Cook, F. V., and W. E. Farrar.** 1978. Vancomycin revisited. Ann. Intern. Med. **88**:813-818.
6. **Crossley, K. B., J. C. Rotschafer, M. M. Chern, K. E. Mead, and D. E. Zaske.** 1980. Comparison of a radioimmunoassay and a microbiological assay for measurement of serum vancomycin concentrations. Antimicrob. Agents Chemother. **17**:654-657.
7. **Geraci, J. E., F. R. Heilman, D. R. Nichols, W. E. Wellman, and G. T. Ross.** 1957. Some laboratory and clinical experiences with a new antibiotic, vancomycin. Antibiot. Annu. **1956-1957**:90-105.
8. **Griffith, R. S.** 1957. Vancomycin: continued clinical studies. Antibiot. Annu. **1956-1957**:118-122.
9. **Jordan, D. C., and P. E. Reynolds.** 1975. Vancomycin. Antibiotics (USSR) **3**:704-718.
10. **Kirchmeier, R. L., and R. P. Upton.** 1978. Simultaneous determination of vancomycin, antisomycin and trimethoprim lactate by high pressure liquid chromatography. Anal. Chem. **50**:348-351.
11. **Leach, W.** 1962. Ototoxicity of neomycin and other antibiotics. J. Laryngol. Otol. **76**:774-796.
12. **Lee, C. C., R. C. Anderson, and K. K. Chen.** 1967. Vancomycin, a new antibiotic. V. Distribution, excretion and renal clearance. Antibiot. Annu. **1956-1957**:82-89.
13. **Marshall, J. F.** 1964. Structure studies on vancomycin. J. Med. Chem. **8**:18-23.
14. **McCormick, M. H., W. M. Stark, G. E. Pittenger, R. C. Pittenger, and J. M. McGurie.** 1956. Vancomycin, a new antibiotic. I. Chemical and biological properties. Antibiot. Annu. **1955-1956**:606-611.
15. **Nars, P. W., and W. M. Hunter.** 1972. A method for labeling oestradiol-17β with radioiodine for radioimmunoassays. J. Endocrinol. **57**:192-193.
16. **Sheldrick, G. M., P. G. Jones, D. Kennard, D. H. Williams, and G. A. Smith.** 1978. Structure of vancomycin and its complex with acetyl-D-alanyl-D-alanine. Nature (London) **271**:223-225.
17. **Smith, K. A., D. H. Williams, and G. A. Smith.** 1974. Structural studies on the antibiotic vancomycin: the nature of the aromatic rings. J. Chem. Soc. Perkin Trans. 1, p. 2369-2376.
18. **Smith, R. M., A. W. Johnson, and R. D. Guthrie.** 1972. Vancosamine. A novel branched chain amino-sugar from antibiotic vancomycin. J. Chem. Soc. Chem. Commun. **00**:361-362.
19. **Torres-R, J. R., C. V. Sanders, and A. C. Lewis.** 1979. Vancomycin concentration in human tissues—preliminary report. J. Antimicrob. Chemother. **5**:475-477.
20. **Uhl, J. R., and J. P. Anhalt.** 1978. High performance liquid chromatographic assay of vancomycin in serum. Ther. Drug Monit. **1**:75-83.
21. **Walker, C. A., and B. Kopp.** 1978. Sensitive bioassay for vancomycin. Antimicrob. Agents Chemother. **13**: 30-33.
22. **Weringa, W. D., D. H. Williams, J. Feeney, J. P. Brown, and R. W. King.** 1972. The structure of an amino-sugar from the antibiotic vancomycin. J. Chem. Soc. Perkin Trans. 1 p. 443-446.