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Short communication

New sensitive assay of vancomycin in human plasma using high-performance liquid chromatography and electrochemical detection

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Abstract

A method using reversed-phase high-performance liquid chromatography with electrochemical detection for the analysis of vancomycin in human plasma was developed. Chromatographic conditions included an octadecyl column, a mobile phase of acetonitrile–sodium phosphate buffer (pH 7) (12:88), a total run time of 12 min, and coulometric electrochemical detection at +700 mV. Linear detector response was found in the range 5–100 μg ml^{-1} after a 1:80 dilution or from 0.5 to 50 μg ml^{-1} after a 1:20 dilution of the samples. In both cases the correlation coefficient (r) of the calibration curve standard was better than 0.995. Vancomycin determination was based on a denaturation of plasma proteins with methanol, then a dilution with mobile phase was performed. Recovery of vancomycin from plasma was 103.1±3.9%, and no interference from commonly used drugs or endogenous compounds was observed. A significant correlation was shown with the EMIT assay (r=0.92, P<0.001) using clinical samples from children. This HPLC technique is simple, sensitive, rapid, precise, selective and requires only 100 μl of plasma for completion.

Keywords: Vancomycin

1. Introduction

Vancomycin, an amphoteric glycopeptide antibiotic isolated from *Streptomyces orientalis*, is a drug used in the treatment of serious gram-positive infections, thus penicillin-resistant, and staphylococci and streptococci. It is also effective against *Corynebacteria* and *Clostridium difficile*. Vancomycin has been used less during past years because of the toxicity of its impure preparation, but this drug has been rehabilitated because of development of methylcillin-resistant bacteria. Vancomycin is known for its potential ear and kidney toxicity at a plasma concentration of 50–80 μg ml^{-1} [1,2]. So, the monitoring of blood vancomycin level is necessary for patient safety. Many methods of vancomycin measurement in body fluids are described. Techniques are either bioassay [3,4], radioimmunoassay (RIA) [4], fluorescence polarization immunoassay (FPIA) [4–9], enzyme multiplied immunoassay (EMIT) [6,9] or high-performance liquid chromatography (HPLC) [2,4,5,7,10,11]. The first technique

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lacks precision [4,5], the second has the same precision default and requires handling of radioisotope materials [10,12], the third presents a vancomycin overestimation caused by a cross reactivity with a crystal degradation product (CDP-1) of vancomycin when polyclonal antibodies are used [4,12], the fourth is not affected by the inactive CDP-1 but it is less accurate and sensitive [6,8,9]. The last method seems to be the most reliable, precise and sensitive compared to the others. Several methods are described using cyano- or aminopropyl bonded phase columns [2,5]; gradient elution mode [10,11]; UV detection at 282 nm [7], 240 nm [2], 225 nm [5] or 214 nm [10]; with a large volume of plasma, necessitating a step of extraction and/or purification on a solid-phase extraction (SPE) cartridge [10,11,13] or directly injecting supernatant after acidic or solvent protein precipitation [2]. Another assay was reported using a fully automated HPLC system [14].

The present report describes a simple, rapid, precise, selective and suitable HPLC assay for the measurement of vancomycin in plasma with a low volume of biological fluid. Electrochemical detection increases the sensitivity of the assay. Moreover, the lifetimes of the analytical columns increase since an extensive dilution of samples is performed.

2. Materials and methods

2.1. Chemicals and samples

Vancomycin and hydrogen disodiumphosphate heptahydrated salt were obtained from Sigma (St. Quentin Fallavier, France). HPLC-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Concentrated orthophosphoric acid was obtained from Carlo Erba. Blank plasma was obtained from a blood bank of healthy volunteers and children samples from the Debrousse hospital's Department of Pediatrics.

2.2. Preparation of standard solutions

Stock solutions of vancomycin were prepared in purified water (2.5 mg ml^{-1}). Solutions were prepared weekly, and stored at 4°C, even though the solution was stable for more than 2 months [15]. An aqueous stock solution was serially diluted to concentrations of 100, 50, 25, 10 and 5 μg ml^{-1} in blank plasma. The volume of water spiking solution added to the blank matrix was always less than 5% of the matrix volume.

2.3. HPLC system and chromatographic conditions

Separation of vancomycin was carried out using a 250×4.6 mm I.D., 5 μm, stainless steel column packed with Kromasil C_{18} (5 μm) (Akzo Nobel) stationary phase. A pre-column (10×4 mm) with the same packing was used. The mobile phase was a mixture of acetonitrile–water (12:88, v/v). It contained 25 m*M* Na_2HPO_4 and was adjusted to pH 7.0 with concentrated orthophosphoric acid. The mobile phase was filtered through a 0.22-μm Whatman filter and degassed by sonication for 10 min before use.

The samples were injected onto the column using a 20-μl loop sample injector (Rheodyne, Model 7125, Berkeley, CA, USA). A flow-rate of 0.8 ml min^{-1} was maintained using a constant-flow pump Model LC-l0AD (Shimadzu, Japan). The electrochemical detection system consisted of an ESA Coulochem II (Bedford, MA, USA) with a dual electrode analytical cell Model 5010 (ESA) and a guard cell Model 5020 (ESA) placed before the injector. Applied electrode potentials were set at +1000 mV for the guard cell and +400 mV and +700 mV for electrodes E1 and E2, respectively. Range was set at 5 μA. The peak areas were integrated by a Shimadzu Model C-R6A Chromatopac integrator. Chromatographic separations were performed at room temperature.

2.4. Sample preparation

A 100-μl volume of plasma was combined with an equal volume of methanol in a 1.5-ml capped microcentrifuge plastic tube and mixed thoroughly on a vortex mixer to denature proteins. A 200-μl volume of mobile phase was added to the mixture and was centrifuged for 5 min at 3000 *g* at room temperature. A 100-μl volume of supernatant was transferred to a 5-ml capped polypropylene tube, and 1.9 ml of mobile phase was added leading to an 80-times dilution of samples. The mixture was mixed

for 10 s and 20 μl of solution was injected onto the HPLC device.

2.5. Interfering substances

Numerous common drugs were tested: acetaminophen, amikacin, amitriptyline, caffeine, cefazolin, chloramphenicol, cimetidine, cortisol, ceftazidime, cephalexin, carbamazepin, desipramine, estriol, fucidine, fosfocin, flecainide, gentamycin, imipramine, imipenem, lidocaine, methotrexate, netilmicin, nortriptylin, propranolol, phenobarbital, primidone, procainamide, quinidine, ranitidine, rifadin, rocephin, salicylate, theophyllin, ticarcillin, tobramycin.

2.6. Validation

The method was validated regarding its accuracy and precision, linearity, limit of quantification (LOQ) and limit of detection (LOD) and recovery.

Accuracy and linearity were assessed with spiked samples ($n=3$) at 5, 10, 25, 50 and 100 μg ml^{-1} of vancomycin on 3 different days. Repeatability of the assay was assessed by performing analyses of spiked samples ($n=6$) at 10, 50 and 100 μg ml^{-1} in plasma against a calibration curve during the same day. Reproducibility was determined with spiked samples ($n=6$) at 10, 50 and 100 μg ml^{-1} in plasma prepared on 3 different days. LOD was determined as the lowest concentration for which the peak area had a signal-to-noise ratio of 3:1. LOQ was determined as the lowest concentration for which the precision in measuring the peak area response was lower than 15%. The recovery of vancomycin from plasma was determined by comparing the slopes from the treated plasma samples with those obtained from untreated aqueous standards of the same concentrations.

The external standard method was used in the calibration and evaluation of the unknown samples.

3. Results and discussion

3.1. Electrochemical detection and interference

The structure of vancomycin permitted its measurement by amperometric detection in oxidative mode. The hydrodynamic voltammogram for vancomycin in chromatographic conditions described above is shown in Fig. 1. Background current and range of interferences were increased using applied potential over +700 mV. So, the working potential to the second electrode was set at this value although the sigmoidal curve plateau was obtained at higher voltage. The voltammogram showed a two-wave line, because vancomycin was composed of various chemical functions with different reduction/oxidation potentials. The applied potential was chosen at +700 mV for better signal-to-noise ratio.

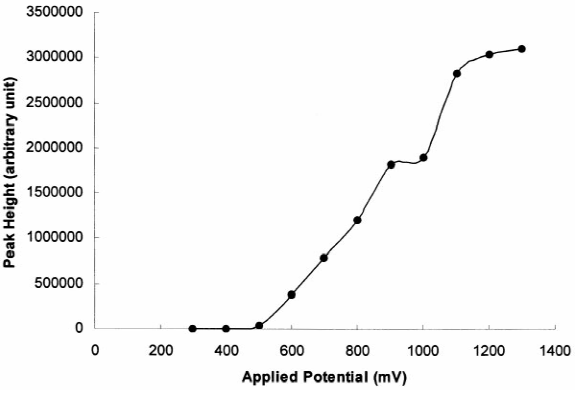

Fig. 1. Hydrodynamic voltammogram of vancomycin (average of duplicate assays). The details of HPLC conditions are described in Materials and methods.

Under the chromatographic conditions described the retention time of vancomycin was 9.7 min. Vancomycin was well resolved and no interference from endogenous plasma components or drugs tested was observed (Fig. 2).

3.2. Linearity and accuracy

The linearity of this assay was confirmed using an analysis of variance (ANOVA). Homocedasticity was statistically determined using Cochran's test ($P=0.95$), so an unweighted least-squares linear regression of the peak area as a function of the theoretical concentrations was applied to each standard curve. The intercept was not statistically different from zero [Student's t-test ($\alpha=0.05$)]. The significance of the slope and the validity of the linear calibration curves were confirmed using Fisher–Snedecor's F-test ($\alpha=0.05$). Correlation coefficient (r) of the linear regression was better than 0.998. The mean recovery from

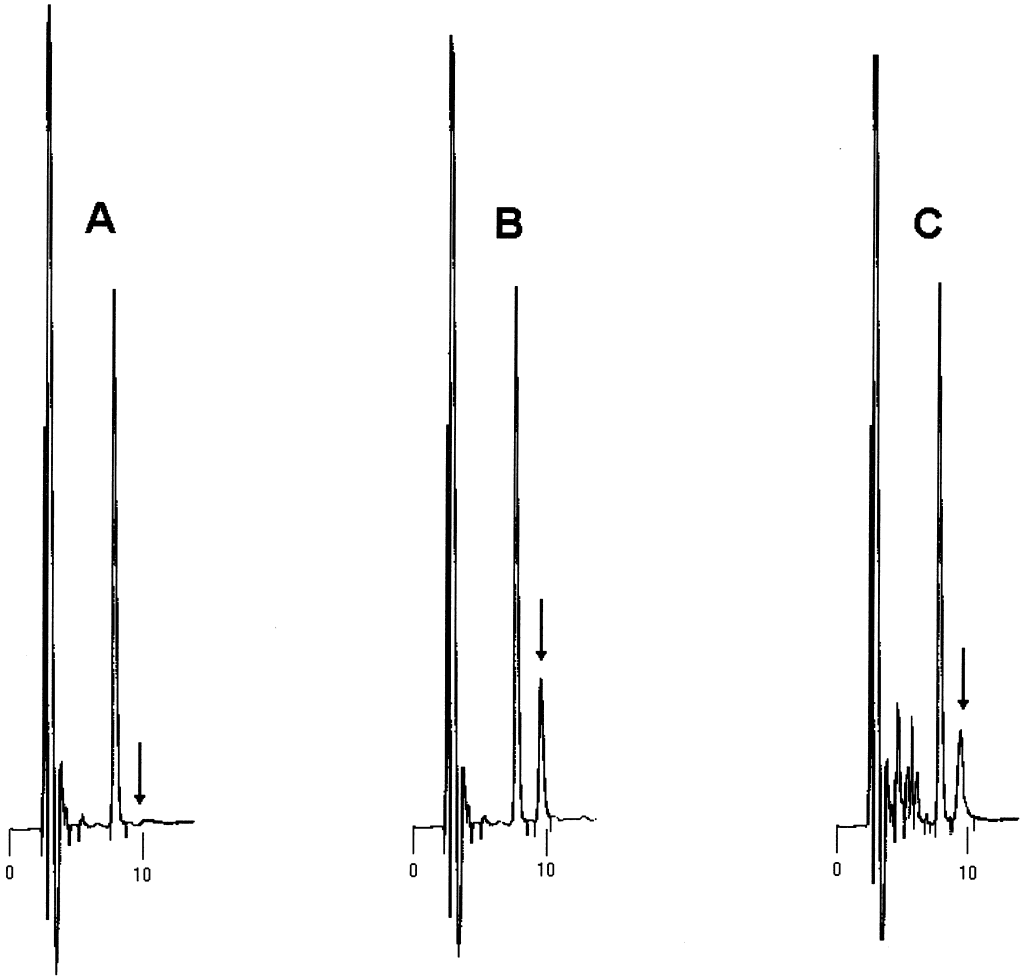

Fig. 2. Chromatograms of a blank plasma (A); blank plasma spiked with 10 μg ml^{-1} (B); sample from children treated with vancomycin (concentration 7 μg ml^{-1}) (C). An arrow indicates the peak of vancomycin.

the five calibration points was 103.1±3.9% [the homocedasticity of variance was tested using Cochran's test (P=0.95)].

3.3. Recovery, precision, LOQ and LOD

The extraction recovery of vancomycin from plasma using methanol as denaturant agent was 91±8.3% (mean±SD). Acetonitrile and perchloric acid were also tested but the extraction recoveries were 33 and 62%, respectively. The LOD was 0.5 μg ml^{-1} and the LOQ was 1 μg ml^{-1}. The results for repeatability and reproducibility are presented in Table 1.

3.4. UV versus electrochemical detection

Numerous studies described the quantification of vancomycin with HPLC and UV detection. Usually, the wavelengths used are in the region of 280 nm [7] or 215 nm [1,10,11], with the latter the sensitivity was dramatically increased. Electrochemical detection permitted an increase of sensitivity. Spiked samples with 10 μg ml^{-1} of vancomycin were analyzed with UV detection (215 nm, AUFS=0.002)

Table 1
Quantification of the precision[a]

Concentration (μg ml^{-1})	RSD (%)	
	Repeatability	Reproducibility
10	2.0	4.2
50	2.0	5.6
100	0.7	4.3

[a] Six injections for each concentration were performed on 1 day and on 3 days for the repeatability and reproducibility, respectively.

and with electrochemical detection ($E=+700$ mV, $R=5$ μA), the signal-to-noise ratios were 65 and 100, respectively.

3.5. Dilution factor

With the intention to decrease the LOQ, the sample was prepared using a dilution of 1:20 instead of 1:80 as described above (100 μl of plasma was used). In this way, the linearity was validated between 0.5 and 50 μg ml^{-1}, with a correlation coefficient over 0.995. The LOQ became equal to 0.25 μg ml^{-1} in plasma. The intra-day repeatability showed a relative standard deviation (RSD) equal to 6.2% and the inter-day repeatability showed an RSD equal to 9.3% with the plasma sample spiked at 0.5 μg ml^{-1} of vancomycin. These results were equal to or better than those obtained with a greater sample volume and using liquid–liquid or SPE [5,7,10,11,13].

3.6. Comparison of HPLC and EMIT assays

A comparison with an EMIT assay (reagent: Dade-Behring; device: Cobas-Mira, Roche) was performed using clinical samples from children treated with vancomycin (and other drugs). Although the available volume of numerous samples was inferior to 50 μl for HPLC assay, analyses were performed as described above keeping the dilution factor to 1:20. The correlation between the two techniques was determined using a linear least-squares regression. A significant correlation was shown ($r=0.92$, $P<0.001$) and the equation of the regression line was: EMIT=1.09(HPLC)−1.25 (Fig. 3). Our results

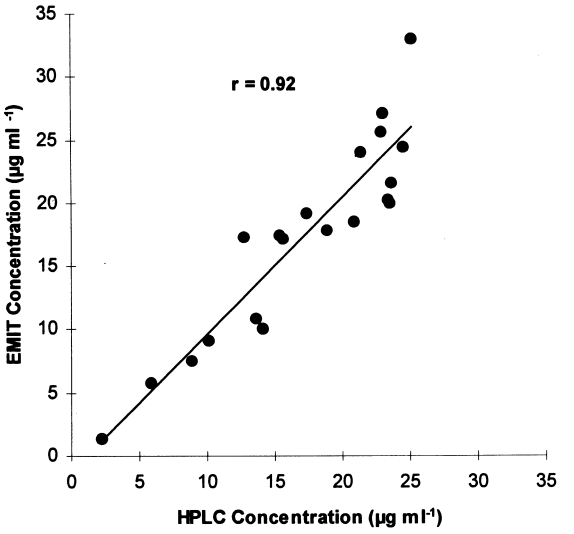

Fig. 3. The HPLC and EMIT correlation from 20 clinical plasma samples.

are similar to those described by Smith et al. [8] [$r=0.87$; EMIT=1.08(HPLC)+2.29] or by Demotes-Mainard et al. [14] [$r=0.96$; EMIT=1.00(HPLC)+0.51].

4. Conclusion

The analytical performances of this HPLC assay for the quantification of vancomycin in human plasma were very good. This method has been validated for concentrations ranging from 5 to 100 μg ml^{-1} after a 1:80 dilution or from 0.5 to 50 μg ml^{-1} after a 1:20 dilution of the samples. This method appears useful because of the small sample volume, the simplicity of sample preparation and lack of interference. Electrochemical detection with oxidation improves the sensitivity and compared to the published method using UV detection, a decrease of the LOQ was reported in the present study. HPLC and electrochemical detection is a convenient method for monitoring of vancomycin in clinical investigations.

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