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Determination of the free and total concentrations of vancomycin by two-dimensional liquid chromatography and its application in elderly patients

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ABSTRACT

A robust two-dimensional liquid chromatography (2D-LC) method for determining the free and total concentrations of vancomycin in plasma was developed and validated. The 2D-LC system, which exhibited a strong capacity for inhibiting interference, comprised a unique RP_1–IEX–RP_2 column system and an "Assistant Flow" configuration. Ultrafiltration technology was employed to separate free vancomycin from the protein-bound fraction in human plasma. The influence of ultrafiltration conditions on the free vancomycin concentration was evaluated. The calibration curve was linear over the 0.195–49.92 μg/ml range for the free and total vancomycin concentrations. The within- and between-run precision ranges were 1.5–3.9% and 2.0–4.7% for the total concentration, 1.4–3.3% and 2.4–4.0% for the free concentration, respectively. Ultrafiltration was susceptible to variations in the experimental conditions, including the centrifugation time, the centrifugal force, and the nominal molecular weight limit of the ultrafiltration membrane. A total of 101 serum samples from 84 elderly patients were analyzed by this method. The free vancomycin concentration was 5.88 ± 3.75 μg/ml (range: 0.240–16.79 μg/ml), the total concentration was 12.36 ± 5.36 μg/ml (range: 2.16–27.14 μg/ml), and the unbound fraction was 45.6 ± 18.8% (range: 11.1–96.9%). There was a poor correlation between the free and total vancomycin concentrations ($R^2 = 0.596$, $p < 0.05$). This method appears to be sensitive, precise, selective, and suitable for use in protein-binding studies of vancomycin.

1. Introduction

Vancomycin, a glycopeptide antibiotic, is widely used in the treatment of serious infections caused by methicillin-resistant *Staphylococcus aureus* [1,2]. Whereas vancomycin underdosing can lead to drug resistance and treatment failure, overdosing may result in nephro- or oto-toxicity. Therefore, therapeutic drug monitoring (TDM) has been extensively recommended to guide the dosing of the drug [3,4]. Generally, TDM focuses on the total drug concentration in human plasma or serum. However, only the "free" (non-protein-bound) portion of the total concentration is responsible for antimicrobial activity. Moreover, the protein binding of vancomycin shows considerable variability across studies [5–12], which may lead to different clinical responses even with the same total concentration. This possibility is especially high for vancomycin, given the narrow trough level of this drug (15–20 μg/ml) that is recommended by recent guidelines [4].

Most studies of vancomycin protein binding have adopted the fluorescence polarization immunoassay technique to determine the free and total drug concentrations [11–16]. However, this technique is unable to recognize crystalline degradation product-1, an inactive metabolite of vancomycin, which can be found in plasma or serum samples from renal-impaired and peritoneal dialysis patients [17–19]. There is limited evidence for using high-performance liquid chromatography (HPLC) to quantify the free and total concentrations of vancomycin. Berthoin utilized [7] two different gradient elutions for separately determining the free and total vancomycin concentrations. However, this approach was time- and labor-consuming, necessitating a complicated pretreatment procedure (evaporation after solid-phase extraction) to determine the total concentration, a long equilibrium interval between runs, and a complex operating procedure.

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Substantial recent interest has focused on the use of two-dimensional liquid chromatography (2D-LC) [20–22], which offers enhanced resolving power and high sensitivity for determining extremely complex, multicomponent mixtures [23–25]. Three different 2D-LC configurations have been reported for detecting the total vancomycin concentration in plasma [26–28]. One configuration employed two reversed-phase (RP) columns (the pre-column and separation column), but the chromatographic peak was poorly resolved [26]. Another system used a short CAPCELLPAK MF cartridge SCX column (10 mm × 4.0 mm) as the first dimension, but failed to resolve the poor separation [27]. A Waters Oasis first-dimensional column equipped with a mass spectrometric detector also generated a series of separation peaks with tailing, distortion, and interference [28].

In the present study, we developed a unique 2D-LC approach to determine the total and free concentrations of vancomycin in human plasma, and optimized the ultrafiltration pretreatment of real samples for the separation of free drug. The developed 2D-LC system included an RP_1–IEX–RP_2 column system and an "Assistant Flow" configuration, without requiring an internal standard. To the best of our knowledge, no previous preliminary study has described the detection of free and total vancomycin in plasma using 2D-LC. We applied the 2D-LC system to analyze the total and free concentrations of vancomycin in blood samples from elderly patients.

2. Materials and methods

2.1. Reagents and chemicals

Vancomycin reference substance was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). Pooled human plasma, used for setting up the experiments, was obtained from the hospital medical examination center. Acetonitrile and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany). Analytical grade ammonium acetate, ammonium phosphate, and ethylene alcohol were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Deionized (18 MΩ/cm) water was generated in-house with a Milli-Q Plus system (Millipore, Bedford, MA).

2.2. Solutions

A standard stock solution of 2.50 mg/ml vancomycin was prepared in a mixed liquor of methanol and ethylene glycol (3:1, V/V) and stored at −20 °C, where it remained stable for at least 3 months. Working solutions of 3.90–998.40 μg/ml vancomycin were prepared by serial dilution from the standard stock solution with 25% ethylene glycol. Working solutions were freshly prepared on each experimental day. For the calibration curve, serial solutions of 0.195, 0.507, 2.03, 4.99, 14.98, 34.94, and 49.92 μg/ml vancomycin were freshly prepared by spiking drug-free plasma (for total concentration) or drug-free deproteinated plasma (for free concentration) with the appropriate amount of working solution. To obtain deproteinated plasma, the plasma was pretreated by ultrafiltration in an Amicon Centrifree Micropartition device with 10-kDa molecular weight (MW) cut-off filters. Quality control (QC) samples were prepared independently in the same way, at concentrations of 0.195 (lower limit of quantification, LLOQ), 0.507 (low), 14.98 (middle), and 49.92 μg/ml (high). All QC samples were dispensed into 2.0-ml capped polypropylene tubes and stored at −70 °C until analysis.

2.3. Chromatographic system

Fig. 1 shows the 2D-LC system, which comprised three parts: the first separation system (LC1), interface, and second separation system (LC2). LC1 consisted of a chromatography pump (LC-20ATvp, Shimadzu, Kyoto, Japan; PUMP1), autosampler device (SIL-20A, 500-μl quantitative loop, Shimadzu; SIL), and LC1 column (first separation column). The interface included 6-port and 10-port switching valves (Rheodyne, Oak Harbor, WA), an assistant high-pressure pump (LC-20ATvp; PUMP2), and two capture columns (A and B). LC2 consisted of a low-pressure gradient chromatography pump with four flow paths (LC-20ATvp; PUMP3), UV detector (SPD-20A, Shimadzu), LC2 column (second separation column), and workstation (LC solution ver. 1.26, Shimadzu).

The LC1 column was an RP column (ASTON C_{18}, 4.6 mm × 100 mm, 5 μm, ANAX, Changsha, China). The mobile phase of PUMP1 was a 90:10 (V/V) solution of 10.0 mmol/l ammonium acetate:acetonitrile (pH adjusted to 3.8 by acetic acid), with a flow rate of 1.0 ml/min. The capture column was an IEX column with a strong ion exchange mechanism (ASTON SCX, 4.6 mm × 20 mm, 5 μm, ANAX). The solution of PUMP2 (Assistant Flow Solution, AS) was an aqueous solution of 10.0 mmol/l ammonium acetate (pH adjusted to 3.0 by acetic acid). The LC2 column used ACR C_{18} (4.6 mm × 250 mm, 5 μm, Shiseido, Japan). The mobile phase of PUMP3 was an 82.5:17.5 (V/V) solution of 50.0 mmol/l ammonium acetate:acetonitrile (pH adjusted to 5.2 by acetic acid), with a flow rate of 1.2 ml/min. The detection wavelength was 282 nm. The "heart-cutting" transfer mode was used between the LC1 and LC2 columns.

A block diagram of the automated analyzer is shown in Fig. 1. The time program of the pump and valve is shown in Table 1.

2.4. Clinical samples

Clinical samples were collected at two teaching hospitals of Central South University: the Second Xiangya Hospital and the Third Hospital of Changsha. All elderly patients (age ≥65 years) who had been treated with vancomycin for a suspected or proven Gram-positive infection and who met the TDM guidelines for vancomycin were included. Clinical vancomycin samples were collected for the TDM of vancomycin as part of the patients' routine care. The serum was split into two aliquots for determining the total and free concentrations. The study was approved by the institution review boards of the two hospitals, and the requirement for informed consent was waived.

2.5. Sample preparation

All vancomycin concentrations (total and free) were determined within 3 h of sample collection. The unbound fraction (f_u) was

Table 1
Time programs for the two-dimensional liquid chromatography method.

Time (min)	Valve 1	Valve 2	PUMP flow (ml/min)		
			PUMP 1	PUMP 2	PUMP 3
0.0–3.50	LC1 linked to waste bottle; SIL injection	Capture column B linked to LC2	1.0	0	1.2
3.51–4.30	LC1 linked to Valve 2	Capture column A linked with LC1	1.0	2.0	1.2
Before next injection	LC1 disconnected from Valve 2	Capture column A captures the analyte	1.0	0	1.2
0–3.50	LC1 linked to waste bottle; SIL injection	Capture column A linked to LC2	1.0	0	1.2
3.51–4.30	LC1 linked to Valve 2	Capture column B linked to LC1	1.0	2.0	1.2