Improved Quantitative Determination of Total and Unbound Concentrations of Six Teicoplanin Components in Human Plasma by High Performance Liquid Chromatography

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Total and unbound concentrations of six teicoplanin components in human plasma were determined by high-performance liquid chromatography with a coextractive cleanup technique. Unbound concentrations of teicoplanin components were estimated after ultrafiltration of plasma. For determination of each component in plasma, plasma was deproteinized with acetonitrile and the supernatant was shaken for 60 s with chloroform under acidic conditions. The recoveries of A3-1, A2-1, A2-2, A2-3, A2-4 and A2-5 were greater than 88%. The within-day and between-day coefficients of variation were 1.3–8.8% and 2.8–11.9%, respectively. The limits of detection in ultrafiltered plasma for each component were 0.82, 2.87, 4.23, 3.36, 7.33 and 4.93 nM, respectively. A good correlation was observed between the FPIA and HPLC methods when total concentrations of each teicoplanin component in patient plasma were determined. The analytical methods established in this study are suitable for determining the total and unbound concentrations of six components of teicoplanin in human plasma and for studying the pharmacokinetics of teicoplanin components in patients.

Key words teicoplanin; component; coextractive cleanup technique; HPLC; unbound concentration

Teicoplanin, glycopeptide antibiotic, is a mixture of six closely related major components of similar polarity and four polar hydrolysis products.¹⁾ The antimicrobial activities of these components against some microbial species are different.²⁾ Pharmacokinetic studies in healthy volunteers have also shown that the plasma protein binding and volume of distribution of these teicoplanin components are different.³⁾ Teicoplanin is highly bound to plasma proteins and low clearance, suggesting that the ratio of total and unbound concentrations in plasma may vary if protein binding changes. Although unbound teicoplanin is thought to be the active species, monitoring of plasma concentrations has been performed using total concentrations of drug. It needs to elucidate the relationship between unbound concentration and clinical efficacy, and the reason for the highly interindividual variations of total and unbound concentrations in plasma.

FPIA is a widely used and very convenient method for determining the plasma concentration of teicoplanin. However, each component of teicoplanin cannot be determined separately and it is difficult to determine unbound concentrations in plasma because of detection limitations. Recently, Cociglio *et al.* reported validated coextractive cleanup techniques for two polar drugs, teicoplanin and ganciclovir.⁴⁾ This approach appears to be simple and time-saving, but they determined only the main component of teicoplanin in their report and in preliminary studies we encountered some problems in applying their method to human plasma.

In this study, we attempted to modify and improve this coextractive cleanup method and apply it to an isocratic HPLC procedure for quantitative determination of each teicoplanin component in plasma and ultrafiltered plasma.

MATERIALS AND METHODS

Chemicals Teicoplanin sodium (lot. no. 72014765) was provided by Aventis Pharma Ltd. (France). The authentic

sample contains 0.98% A3-1, 3.93% A2-1, 56.4% A2-2, 5.96% A2-3, 13.94% A2-4, and 13.94% A2-5, as determined by the HPLC method established in this study. Acetonitrile and chloroform (both HPLC grade) were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Ultrafiltered blank plasma was obtained by centrifugation of human serum (10 min, 3000 *g*, 37 °C) with Ultrafree-MC membrane filters (MW cut-off 30000; Millipore, Bedford, MA, U.S.A.). The adsorption of teicoplanin onto the membrane was negligible. Water used for mobile phase and extraction procedures was purified by a Millipore Milli-Q water filtration system (Millipore, MA, U.S.A.).

HPLC Conditions The HPLC system consisted of a NANOSPACE NI-1 pump and spectrometric detector from Shiseido Co. Ltd. (Tokyo, Japan), a UNI-1 NOISE CLEAN from UNION Co. Ltd. (Osaka, Japan), and a C-R6A Chromatopac integrator and SIL-10Advp auto injector from Shimadzu (Kyoto, Japan). Teicoplanin was detected at a wavelength of 220 nm with a range of 0.001 AUFS. Separation of teicoplanin components with an L-column ODS (particle size: 5 μ m, 12 nm; 250 $\times$ 4.6 mm I.D.; Chemical Evaluation and Research Institute, Japan) was performed at 45 °C. The mobile phase consisted of acetonitrile/0.05 M potassium phosphate buffer (pH 4.0) (23 : 77 (v/v)) pumped at a constant rate of 0.8 ml/min. A Guard-Pak filter (Waters, Milford, MA, U.S.A.) was used as the guard column.

Coextractive Cleanup Procedure Ten microliters of teicoplanin solution in distilled water (50, 100, 200, 400 μ g/ml for plasma; 5, 10, 20, 50 μ g/ml for ultrafiltered plasma) was added to 90 μ l of human plasma or ultrafiltered plasma. The coextractive cleanup procedure was performed according to the method of Cociglio *et al.*⁴⁾ with a slight modification. For plasma, distilled water (0.2 ml) was added to the constituted plasma (0.1 ml), the mixture was stirred with a vortex mixer for 90 s, and then 0.3 ml of acetonitrile was added to precipitate plasma proteins. After centrifugation (5 min, 3000 *g*,

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room temperature), 0.5 ml of the deproteinized supernatant was transferred to another polyethylene tube and 10 μ l of 2 M HCl and 0.4 ml of chloroform were added, and then vortex mixing and centrifuged (5 min, 3000 *g*, room temperature).

For determination of unbound concentration, 0.3 ml of acetonitrile was added to 0.1 ml of the ultrafiltrate. After centrifugation (5 min, 3000 *g*), 0.25 ml of the mixture was transferred to another polyethylene tube and acidified with 5 μ l of 2 M HCl. Finally, the mixture was added to 0.2 ml of chloroform and then vortex mixing and centrifuged (5 min, 3000 *g*). The obtained aqueous layer (0.1 ml) was injected directly into the HPLC system described above.

The obtained peak height was compared to those obtained from standard solution prepared described above for determining the recovery with correction of aqueous volume alteration by inulin concentration added.⁵⁾ The teicoplanin components corresponding to each peak were identified according to the LC-MSMS method previously reported by Ackermann *et al.*⁶⁾ The components of each peak eluted were identified by the results of the monoisotopic mass values and compared to those reported.⁶⁾

Plasma (106 samples) withdrawn for routine therapeutic drug monitoring of teicoplanin in patients receiving treatment with teicoplanin (200–400 mg/d) was used for determination of the drug concentration by the HPLC method established in this study. The teicoplanin concentration was also determined by the fluorescence polarization immunoassay kit (Targocid TDM kit-IBL®, Oxis International Inc. Portland, U.S.A.).

Data Analysis The molar concentrations of teicoplanin components were calculated on the basis of their molecular weights (1563.4 A3-1, 1876.5 A2-1, 1878.6 A2-2, 1878.6 A2-3, 1892.6 A2-4 and 1892.6 A2-5) with the assumption that the absorption coefficients of the components are identical. Specifically, the relative ratios of the areas of the components in the HPLC chromatogram of the authentic sample were assumed to correspond to their molar ratios. The concentrations of teicoplanin components in the authentic sample (1.0 mg/ml) were estimated as 6.25 nM for A3-1, 21.0 nM for A2-1, 300 nM for A2-2, 31.7 nM for A2-3, 73.7 nM for A2-4 and 75.9 nM for A2-5. Therefore, the total teicoplanin concentration was represented as μ g/ml but the concentration of each component was represented as μ M or nM. The recovery was calculated by the corresponding peak area compared to those of standard solution with correction of volume alteration by inulin concentrations.⁵⁾

The relationship between the total concentrations determined by HPLC and FPIA was analyzed by linear regression.

RESULTS AND DISCUSSION

Several analytical methods for determining the plasma concentration of teicoplanin have been reported. Although FPIA is routinely used in clinical situations because it is simple and rapid, it does not allow the separate determination of each component of teicoplanin. Furthermore, because of detection limitations, it is difficult to use the FPIA method to determine unbound concentrations of teicoplanin in plasma. HPLC methods can solve these problems because of their better sensitivity and greater cost effectiveness. Solid-phase extraction and affinity chromatography both involves some-

Fig. 1. Typical Chromatograms of Teicoplanin Used to Spike Human Blank Plasma

(A)–(C) represent chromatograms of teicoplanin standard solution (1 μ g/ml), human blank plasma and teicoplanin (1 μ g/ml) spiked in the same blank, respectively.

what complex procedures, are costly and furthermore result in relatively low recoveries of some components because of their hydrophilicity.

In the coextractive cleanup method, an organic solvent is used to extract interfering materials from plasma rather than the drug itself. Interfering peaks were observed at the time of 5–20 min (The peaks appeared in plasma interfered the peaks of A3-1 and A2-1) when chloroform was used as the cleanup solvent for the plasma. Therefore, we first examined the properties of the interfering peaks observed after chloroform extraction of both acidic and alkaline aqueous layers. The interfering peaks disappeared when chloroform was shaken with the aqueous solution under acidic conditions (Fig. 1) but not under alkaline conditions (data not shown), indicating that the interfering substances in plasma are weakly acidic. On the other hand, the lower the pH of the solvent, the higher the peak area of each teicoplanin component. The peak area of A2-2 when teicoplanin was dissolved in pH 3 buffer was 2-fold higher than that with pH 6 buffer. This may be resulted from the pK_a of teicoplanin (3.1, 7.1).

Another problem was low recovery (35–50%) of teicoplanin from human plasma. As teicoplanin is highly bound to plasma proteins, deproteinization with acetonitrile may be the cause of the low recovery. Recovery improved to reasonable values (72–93%) when the plasma was diluted with distilled water before the addition of acetonitrile. We decided to add a two-fold volume of water to the plasma.

Recoveries and within-day and between-day coefficients of variation of our method after the modifications described above are shown in Tables 1 and 2. The recovery of each component from plasma was higher than 88% and C.V. values were below 12%.

The limits of detection of the components, as estimated by S/N ratio=3, were ranged between 0.82 and 7.33 nM, and the difference may be resulted from the difference of the peak height of components. Calibration curves used for quantification of each component in human plasma and ultrafiltered plasma exhibited excellent linearity, with correlation coefficient $r > 0.998$.

In order to confirm the correlation between the HPLC method developed in this study and the FPIA method routinely used in the clinical setting, the concentrations of teicoplanin in plasma from patients administered teicoplanin

Table 1. Analytical Precision and Recoveries of Teicoplanin Components in Human Plasma

Component	Concentration range in plasma (μM)	Within-day CV (%)	Between-day CV (%)	Recovery (%)
A3-1	0.03—0.25	1.9—8.8	4.8—11.9	89—99
A2-1	0.11—0.84	3.3—6.0	5.4—10.6	89—102
A2-2	1.50—12.0	2.1—6.0	2.8—3.7	95—102
A2-3	0.16—1.27	1.9—4.3	7.2—8.5	108—115
A2-4	0.37—2.95	1.3—5.3	2.8—7.0	94—103
A2-5	0.38—3.04	1.9—3.5	4.5—9.3	88—102

Recovery and within-day CV were obtained from five different sample preparations. Between-day CV was obtained from four different sample preparations on four days.

Table 2. Analytical Precision and Recoveries of Teicoplanin Components in Ultrafiltered Plasma

Component	Concentration range in ultrafiltered plasma (nM)	Within-day CV (%)	Between-day CV (%)	Recovery (%)
A3-1	3.12—31.2	1.8—5.9	3.5—9.5	95—111
A2-1	10.5—105	1.5—5.9	0.5—6.2	98—106
A2-2	150—1500	1.7—2.5	0.6—8.3	97—102
A2-3	15.9—159	2.7—6.2	0.3—4.4	93—97
A2-4	36.8—368	3.1—5.9	0.5—7.3	92—103
A2-5	37.9—379	2.6—4.1	0.7—9.4	92—99

Recovery and within-day CV were obtained from five different sample preparations. Between-day CV was obtained from four different sample preparations on four days.

Fig. 2. Scatter Plot of Paired Total Teicoplanin Concentrations Determined by FPIA and HPLC

Total concentration was calculated by the sum of six components determined by HPLC. The line represents $\text{HPLC} = 1.046 \times \text{FPIA} - 0.453$, $r = 0.864$ ($n = 106$).

Fig. 3. Typical Time Courses of Unbound Concentration of Teicoplanin Components in Plasma after Administration of Teicoplanin (400 mg)

were determined. The concentrations of all components were summed on a molar basis and plotted against the concentration determined by FPIA (Fig. 2). A good correlation was observed between the total concentrations obtained from the FPIA and HPLC methods.

Finally, we applied this method for determining unbound concentrations of teicoplanin components in plasma (Fig. 3).

The analytical methods established in this study are suitable for determining the total and unbound concentrations of teicoplanin components in human plasma and for studying the pharmacokinetics of teicoplanin components in patients.

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