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DETERMINATION OF TEICOPLANIN IN HUMAN PLASMA AND URINE BY AFFINITY AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive, highly selective and simple high-performance liquid chromatographic method for the determination of teicoplanin, a novel glycopeptide antibiotic, composed of six components, in human plasma and urine is described. After an isolation step by affinity chromatography, the antibiotic substances were chromatographed on a Nucleosil C_{18} column with phosphate buffer–acetonitrile according to a gradient profile. All the components were detected by their UV absorption at 240 nm. The concentration of teicoplanin was determined by using the external standard procedure. This

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method was applied to the sum of the six major components as well as to each of them separately. The linearity of the method was checked between 0.5 and 50 μg/ml for plasma and between 2 and 50 μg/ml for urine. The limit of detection was 0.1 μg/ml for both biological fluids. The coefficients of variation of the between-day assays did not exceed 8.6 and 8.9% in plasma and urine, respectively. The application of the method to a pharmacokinetic study of teicoplanin after a single intravenous therapeutic dose in a patient is reported. This rapid technique also appears to be suitable for drug monitoring.

INTRODUCTION

Teicoplanin, the recommended international non-proprietary name for teichomycin [1] (formerly known as teichomycin A_2), is a new glycopeptide antibiotic obtained as a fermentation product from *Actinoplanes teichomyceticus* [2, 3]. It belongs to the class of vancomycin–ristocetin [3], and its antimicrobial spectrum covers most gram-positive microorganisms [4–11]. This antibiotic is a mixture of several components, the six major ones being classified, according to their chromatographic elution order on a reversed-phase column, as A_3 and group A_2 which has five components [12]. These six components are glycopeptide analogues, which have the same core glycopeptide formed by an aglycone that is a linear peptide composed of unusual aromatic amino acids oxidatively linked, and by D-mannose and D-glucosamine as sugars [13, 14]. Fig. 1. describes the structures of the components. A_3 is the core glycopeptide, and group A_2 consists of five components containing an additional N-acyl-D-glucosamine and differentiated by the different acyl aliphatic chains as shown in the figure [15].

It has been established that (i) the mechanism of action of teicoplanin is the inhibition of the biosynthesis of the bacterial cell wall by interfering with the mucopeptides terminated by the dipeptide D-alanyl-D-alanine (D-Ala-D-Ala) [16], and (ii) the central heptapeptide aglycone represents the chemical structural feature specifically responsible for the activity by forming a stable 1:1 complex with the transient structure of the cell wall terminated with D-Ala-D-Ala, while the peripheral carbohydrate moieties apparently do not significantly influence the intrinsic activity but have a role on the overall activity in the living cell [17, 18].

Because teicoplanin has greater antibacterial activity than other agents [4–8, 10, 19], this new drug is currently being thoroughly studied as a β-lactamase-resistant agent for the treatment of human infections caused by the pathogens mentioned above. Consequently, several pharmacokinetic studies have been performed [11, 20, 21] using a microbiological assay for the determination of teicoplanin in biological fluids. However, such a technique may not be specific because bacteriologically active metabolites or degradation products might interfere with the determination of teicoplanin. Preliminary data from a liquid chromatographic (LC) method with fluorescence detection for the determination of teicoplanin in serum were recently presented [22].

The aim of the present work was to develop a new specific method to determine the concentrations of total teicoplanin and of each of its main in biological fluids. This was achieved by using an isolation step by the affinity method [23] followed

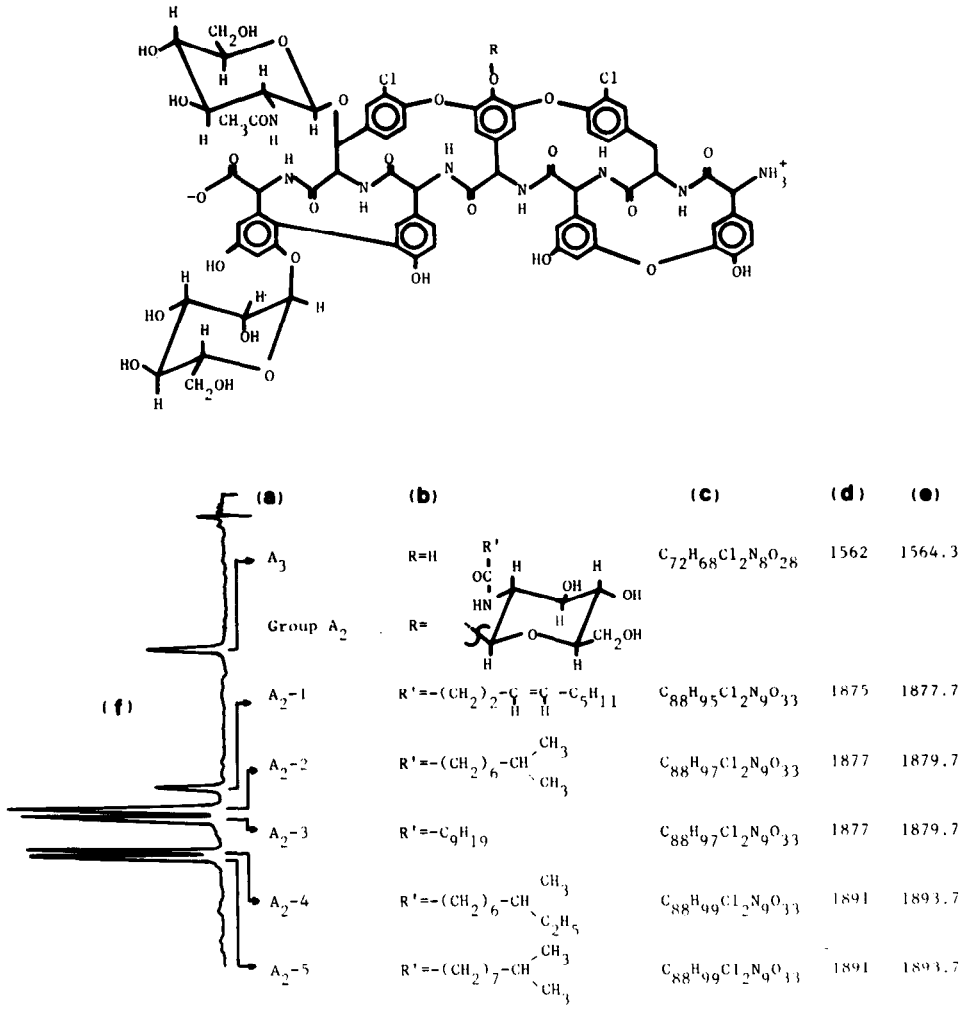

Fig. 1. Structures of the teicoplanin components and their relationship to the HPLC chromatogram. (a) Component name; (b) structural formula; (c) molecular formula; (d) relative molecular mass estimated by mass spectrometry; (e) calculated relative molecular mass; (f) HPLC.

by a separation step by reversed-phase chromatography and by UV detection. A pharmacokinetic study, reported as an application of this method, demonstrates the high specificity of this technique and its utility for monitoring the drug in patients with impaired renal function.

EXPERIMENTAL

Chemicals

Teicoplanin analytical reference substances (ARS) was supplied, as its sodium salt, by Lepetit Research Centre (Milan, Italy), and contained 894 μg/mg of pure teicoplanin. Acetonitrile (Carlo Erba, Milan, Italy) was of HPLC grade, and all

other reagents were of analytical grade. Ammonium hydroxide (15%; Riedel de Haën, Hannover, F.R.G.), prepared as required, and hydrochloric acid (18%; Merck, Darmstadt, F.R.G.) were obtained by dilution with twice-distilled water. D-Alanyl-D-alanine-ε-aminocaproyl Sepharose CL-6B was used as affinity resin. Prepacked columns (0.5 ml of resin volume) were available from Lepetit Research Centre, and were stored in 0.1 *M* Tris buffer (pH 8.5) containing 0.004% sodium merthiolate as preservative. Before use, the affinity columns were equilibrated with 0.05 *M* sodium dihydrogenphosphate–0.2 *M* sodium chloride buffer adjusted to pH 7.5 with 1 *M* sodium hydroxide. When containing 0.004% of sodium merthiolate, this equilibrating buffer was used as a washing solution to rinse the columns between each assay.

Standard solutions.

A stock solution was prepared by dissolving teicoplanin ARS in the equilibrating phosphate buffer (pH 7.5) to obtain an exactly known concentration corresponding to ca. 100 μg/ml of pure substance. When stored at 4°C this solution was found to the stable for 1 month at least. To study the linearity of the method, working solutions (0.5–50 μg/ml) were prepared as needed by diluting the stock solution in the same solvent. In routine analysis the calibration standards for plasma and urine were prepared as required by spiking the corresponding biological fluid with the stock solution to obtain a final concentration of 20 μg/ml (external standard).

Apparatus

The chromatographic analyses were performed with an automated system consisting of two Model 414 high-pressure pumps (Kontron, Zürich, Switzerland) with, for one of them, a Model 810 pulse damper (Kontron), a Model 802 mixing chamber and a Model MSI 660 autosampler (Kontron) equipped with a 170-μl sample loop. The components were detected at 240 nm by using a Model Uvikon 720 LC (Kontron) variable-wavelength detector (2-nm slit). The chromatographic system was automated with an Anacomp 220 computer (Kontron) for sampling control, flow programming, data recording and integration. The whole system was kept in an air-conditioned room (22±1°C).

Chromatographic conditions)

The analytical (10 cm×4.6 mm I.D.) and guard (3 cm×4.6 mm I.D.) stainless-steel columns were packed with Nucleosil C_{18}, particle size 5 μm (Macherey-Nagel, Düren, F.R.G.) according to the ascending slurry-packing technique described by Bristow et al. [24]. The mobile phase was a mixture of two solvents: solvent A was 0.01 *M* sodium dihydrogenphosphate (pH 4.9) and solvent B consisted of acetonitrile–solvent A (50:50). Before use, these solvents were degassed in an ultrasonic bath, and the mobile phase was delivered at a flow-rate of 1.3 ml/min producing a pressure of 120 to 150 bar according to the following gradient profile:

Time (min)	0	0.5	44.5	46.5	48	53	55	60
% B	22	22	53	53	100	100	22	22

In order to maintain the condition of the column the flow-rate was kept at 0.2 ml/min with 22% of B during the stand-by periods.

Extraction procedure

Teicoplanin was isolated and quantitatively recorded from biological samples (urine or plasma) by affinity chromatography, which consisted of the selective binding of teicoplanin to a matrix bearing the group D-Ala-D-Ala previously immobilized through ε-aminocaproic acid to Sepharose [23].

Affinity columns were equilibrated by percolation with 10 ml of phosphate buffer (pH 7.5); no column which showed a percolation time longer than 15 min was used. The head of the equilibrated column was loaded with 2 ml (according to the expected concentration of teicoplanin, a sample of 1–10 ml could be assayed) of sample (standard solution, spiked biological fluid used as external standard, plasma or urine to be assayed). The eluate was discarded, and the resin was washed with 1 ml of phosphate buffer (pH 7.5). Teicoplanin was desorbed from the affinity column by eluting three times with 0.5 ml of 1.5% ammonium hydroxide. The effluents were collected in a 5-ml glass tube, and the mixture was made acidic by adding 0.1 ml of 18% hydrochloric acid. These solutions have proved stable for at least 24 h when stored at room temperature (20–21°C) and for 1 week at 4°C. Afterwards, the affinity column was washed with 5 ml of phosphate buffer (pH 7.5) containing 0.004% of sodium merthiolate as preservative, and stored at 4°C. Under these conditions a single column could be used three to five times, provided the restriction concerning the percolation time was adhered to.

A 170-μl volume extract (injector loop) was injected onto the analytical column in order to ensure the separation of major components of teicoplanin by using the elution gradient described.

Calculations

The concentration of teicoplanin in biological samples was determined by using the external standard procedure. With the assumption that each component of teicoplanin gives an identical response to the UV detection, this method was applied to the sum of the six major components as well as to each of them separately. The amount of total teicoplanin present in the sample was calculated as follows:

$$\text{Total teicoplanin, } \mu\text{g/ml} = \frac{\Sigma A_s \times C \times V_{std}}{\Sigma A_{std} \times V_s}$$

where ΣA_s = sum of the areas of the peaks of the six major components of teicoplanin in the sample to be assayed; C = concentration (μg/ml) of teicoplanin in the spiked biological fluid used as external standard; ΣA_{std} = sum of the peaks of the six major components of teicoplanin in the external standard; V_{std} = volume of spiked biological fluid used to prepare the external standard; V_s = volume of the sample assayed.

The amounts corresponding to each of the six major components were calculated from the ratio of the area under each individual peak to that obtained in the external standard for the six major components. However, because the six main

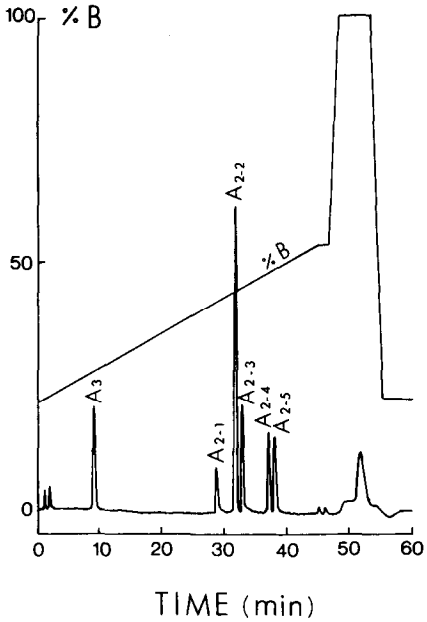

Fig. 2. Typical chromatogram obtained from a standard solution of teicoplanin (100 μg/ml pure substance), and the gradient profile (% B) used for the elution of components, column clean-up and reequilibration.

components represent together ca. 93% of the total teicoplanin, the amount of a particular component previously determined was corrected by a factor accounting for the participation of each of six major components according to the data (see Results) obtained from the mean of fifteen injections of the standard solution.

RESULTS

Chromatographic data

A typical chromatogram obtained from the standard solution (Fig. 2) shows that teicoplanin is constituted of about eight components (A_3, group A_2 plus minor components). Their separation was obtained by using a gradient profile of mobile phase as shown (% B) in the figure. After complete elution of teicoplanin (ca. 46 min), the percentage of solvent B was increased to 100% and maintained for 5 min to clean the column, then B decreased to its initial value (22%) for 5 min reequilibrate to the column. It must be noted that, for all the six major components, the resolution is satisfactory considering the large number of very similar compounds to be separated within a reasonable analysis time. The most important chromatographic characteristics of the main peaks are given in Table I. The symmetry factors found between 1.6 and 1.9 indicate the use of the peak areas for the calculations. In addition, as indicated previously (see *Calculations*), from the mean of fifteen injections of this standard solution of pure teicoplanin the percentage distribution of the six major components was estimated. The results in Table II were used to calculate the individual concentrations of these main components.

TABLE I

CHROMATOGRAPHIC PARAMETERS OBTAINED FOR THE MAIN COMPONENTS OF TEICOPLANIN

Component	Retention time (min)	Capacity factor	Symmetry factor*
A3	9.1	6.0	1.7
A2-1	28.8	21.2	1.9
A2-2	31.6	23.3	1.7
A2-3	32.8	24.2	1.8
A2-4	37.1	27.5	1.6
A2-5	38.0	28.2	1.7

*Determined at 10% of the peak height from the base.

TABLE II

PERCENTAGE OF THE SIX MAJOR COMPONENTS OF TEICOPLANIN OBTAINED OVER FIFTEEN INJECTIONS OF PURE SUBSTANCE (STANDARD SOLUTION)

Component	Percentage* (mean ± S.D.)
A3	11.52 ± 0.43
A2-1	5.42 ± 0.32
A2-2	40.32 ± 0.57
A2-3	13.67 ± 0.29
A2-4	11.37 ± 0.34
A2-5	10.21 ± 0.35
Sum	92.57 ± 0.78

*Percentage of the total areas under all the peaks of teicoplanin.

Chromatograms obtained for blank plasma and urine before and after spiking with known amounts of teicoplanin, and for biological samples from a patient who received 3 mg/kg i.v. teicoplanin, are shown in Fig. 3.

No interference from endogenous substances in the blank samples was observed in the elution range of teicoplanin, but (especially with urine) it was necessary to clean the column before the next injection. The cleanliness of the samples was due to the use of a specific affinity chromatography step before the partition chromatography.

Linearity

For each biological fluid, a standard curve was generated by spiking samples of the corresponding fluid with various amounts of teicoplanin (concentration ranges 0.5–5 μg/ml and 2–50 μg/ml for plasma and 2–50 μg/ml for urine) and analysing them by this method. In the case of plasma, the linearity was carefully checked in the range of low concentrations to make sure that the determination of plasma levels during the elimination phase of the drug could be precisely obtained in pharmacokinetic studies. In each case a linearity test was applied to the data

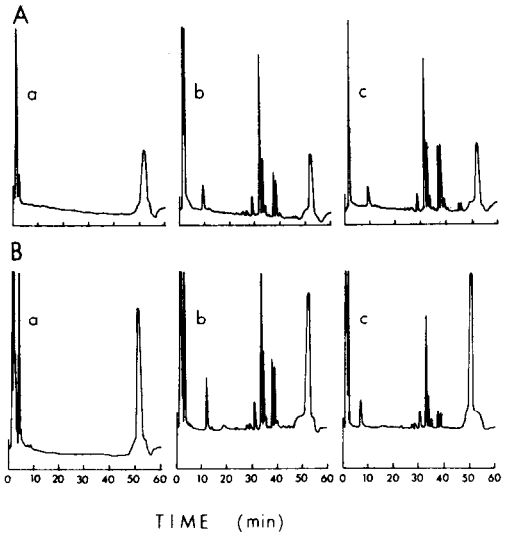

Fig. 3. Chromatograms obtained from extracts of (A) plasma and (B) urine. (a) Controls; (b) samples with 20 μg/ml of teicoplanin used as external standard; (c) samples from a patient who had received a single i.v. dose of 3 mg/kg of teicoplanin 1 h (plasma) and 8 h (urine) before sampling.

obtained from five assays for each concentration studied, and a linear relationship was found between the sum of areas under the six major peaks and the teicoplanin concentration. The results in Table III indicate a high value of the regression coefficients associated with a relatively low value of the intercept, which confirms the lack of endogenous interference, as already shown in Fig. 3. However, it must be noted that the slopes of the regression lines were not similar between assay series (plasma and urine concentration ranges), and the standard deviation of the slope reached 10%. These variations were caused by the slight changes in chromatographic conditions frequently needed since the lifetime of the columns was short (see Discussion). As a consequence, for each sample series, a spiked biological fluid was used as an external standard to form the basis for the quantitation.

TABLE III

PARAMETERS OF LINEAR STANDARD CURVES

$n = 5$ for all determinations.

Biological fluid	Teicoplanin concentration range (μg/ml)	Slope (mean ± S.D.)	Intercept (mean ± S.D.)	Regression coefficient, r^2
Plasma	2 -50	56.89 ± 2.45	5.39 ± 2.18	0.9986
Plasma	0.5- 5	36.45 ± 4.12	2.23 ± 1.55	0.9983
Urine	2 -50	47.71 ± 2.46	−0.97 ± 0.46	0.9988

TABLE IV

RECOVERY OF TEICOPLANIN FROM PLASMA AND URINE

$n = 6$ for all determinations.

Biological fluid	Teicoplanin concentration (μg/ml)	Recovery (mean ± S.D.) (%)	C.V. (%)
Plasma	2	94.5 ± 0.5	0.53
	20	94.2 ± 0.7	0.74
Urine	2	93.8 ± 0.4	0.43
	20	93.1 ± 0.5	0.53

TABLE V

BETWEEN- DAY PRECISION OF TEICOPLANIN MEASUREMENT IN SPIKED PLASMA AND URINE

$n = 6$ for all determinations.

Biological fluid	Amount of teicoplanin added (μg/ml)	Amount of teicoplanin found (mean ± S.D.) (μg/ml)	C.V. (%)
Plasma	0.5	0.56 ± 0.03	6.5
	1	1.01 ± 0.02	1.4
	2	1.96 ± 0.16	8.6
	5	5.05 ± 0.08	1.6
	20	20.33 ± 0.96	4.8
Urine	2	2.01 ± 0.18	8.9
	20	20.09 ± 1.16	5.8
	50	50.18 ± 2.05	4.1

Recovery

The overall recovery was determined by comparing the sums of the areas under the six major peaks from directly injected standard solutions with those from spiked biological fluid solutions taken through the whole procedure. The results in Table IV show that the recovery of teicoplanin was excellent (ca. 94%) for plasma and urine, with low coefficients of variation (C.V.). Identical results were obtained in both the low (2 μg/ml) and the high (20 μg/ml) concentration ranges for both biological fluids.

Limit of detection and precision

Under the experimental conditions described, the limit of detection, based on a signal-to-noise ratio of 3:1, was 0.1 μg/ml for plasma and urine. For this concentration, only the peak corresponding to the component A2-2 was measurable. This limit might be increased by using a volume of biological fluid larger than 2 ml without modifying the procedure.

The precision of the assay was established by replicate analysis of samples over the concentration range defined for the study of linearity, which represents the

entire range of teicoplanin levels currently encountered after a i.v. therapeutic dose in humans. For both biological fluids studied, the within-day precision on six spiked teicoplanin samples of each fluid determined was 6.8% and 4.4% in plasma and 6.2% and 4.4% in urine for concentrations of 2 and 20 μg/ml, respectively. The between-day C.V. did not exceed 8.6% in plasma and 8.9% in urine (see Table V).

DISCUSSION

As stated in the introduction, only microbiological methods were previously used for the determination of teicoplanin in biological fluids [11, 20, 21]. However, this technique did not meet all the requirements for an accurate pharmacokinetic study because the metabolism and the antibacterial activity of the components of teicoplanin can differ. Only recently, preliminary information on a chromatographic method became available [22].

The method described here is relatively simple and rapid, and can be applied to determine accurately the concentration of total teicoplanin or of its main components in plasma and urine. The high specificity of this method relies the extraction step, involving the use of an affinity resin that was developed by attaching D-Ala-D-Ala to a complex support such as agarose, cellulose or sepharose through a spacer arm formed by condensation of ε-aminocaproic acid and cyanogen bromide. Interestingly, such a specificity also prevents interference from several drugs that could be administered with teicoplanin. Such a lack of interference has been checked for diuretics (furosemide, hydrochlorothiazide), β-blockers (atenolol, oxprenolol, acebutolol), analgesics (aspirin, paracetamol), hypolipidemic agents (clofibrate, benfluorex) and tranquillizers (chlorazepam, diazepam, nitrazepam).

Owing to its automation, the stability of the chromatographic system was found to be highly satisfactory as only changes in the retention times could be observed over 6 months. These slight modifications were due to the changes of guard and analytical columns, which were not packed with the same batch of stationary phase within this routine work period. Indeed, under the chromatographic conditions described, the column lifetime was found to be short since the guard and analytical columns had to be changed each 30 and 60 assays, respectively. In order to improve this lifetime, a column clean-up was attempted by using solvents such as acetonitrile, methanol and buffer at pH lower than 3. When this approach failed, C_{18} stationary phases with a particle size of 10 μm (Nucleosil, Spherisorb ODS-II from Phase Separations, Norwalk, NJ, U.S.A.) were tested. They slightly increased the lifetime of the columns, but the use of 10 μm particles was not suitable as the resolution and symmetry factors were significantly altered. It was necessary to use two spiked samples as external standards with every batch of samples to be analysed in order to ensure the reliability of the method (see Table V).

This HPLC method was compared with the microbiological assay (Gruppo Lepetit, Milan, Italy) by the simultaneous analysis of biological samples (obtained from patients). A close relationship was found between the two sets of data for total teicoplanin ($r=0.97$, $n=354$, $p<0.001$ for plasma and $r=0.99$, $n=174$,

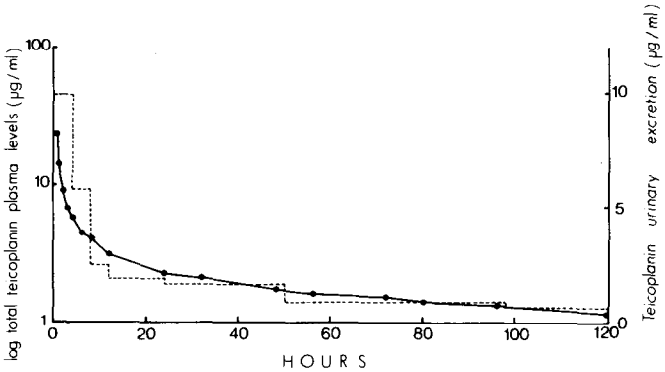

Fig. 4. Time-course of plasma levels (●) and urinary excretion (---) of teicoplanin (as the sum of the six main components) in a patient with impaired renal function given 3 mg/kg i.v. of teicoplanin at 8.00 a.m. (pharmacokinetic parameters: elimination half-life = 123 h; concentration at time zero = 32 µg/ml; area under the curve = 465 µg h ml^{-1}; residence time = 154 h; volume of distribution at the steady-state = 0.99 l kg^{-1}; total clearance = 0.0065 l h^{-1} kg^{-1}).

$p < 0.001$ for urine). When only the samples that exhibited, using the HPLC technique, a total teicoplanin concentration below 2 µg/ml were considered, this relationship remained highly significant but was slightly less close ($r = 0.86$, $n = 171$, $p < 0.001$ and $r = 0.79$, $n = 56$, $p < 0.001$ for plasma and urine, respectively.

The limit of detection of the method was similar to that obtained with the microbiological assay, and thus appears suitable to determine accurately the plasma and urine concentrations in pharmacokinetic studies. As an example, Fig. 4 shows the concentrations of teicoplanin observed in plasma and urine from a patient who had received a therapeutic dose (3 mg/kg i.v.) of teicoplanin. The time-course of the concentration of teicoplanin in plasma showed a triphasic decline and could be fitted as the sum of three exponentials. As the half-life elimination was very long (more than 100 h), it must be noted that the method allowed the measurement of plasma concentrations up to 120 h after administration.

In conclusion, the method described is highly selective, sensitive and reliable. It was demonstrated to be suitable for the measurement of teicoplanin concentrations in biological fluids in humans who have received a single i.v. therapeutic dose. Therefore, it can be used to determine the pharmacokinetic parameters of total teicoplanin and of its components, separately, and their possible alteration in pathological states such as renal insufficiency. Finally, this rapid method (20 samples can be assayed every day) might be used for drug monitoring in hospitals.

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