Determination of Teicoplanin Concentrations in Serum by High-Pressure Liquid Chromatography

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An isocratic reversed-phase high-pressure liquid chromatographic method for the determination of six components of the teicoplanin complex in biological fluid was developed. By using fluorescence detection after precolumn derivatization with fluorescamine, the assay is specific and highly sensitive, with reproducibility studies yielding coefficients of variation ranging from 1.5 to 8.5% (at 5 to 80 μg/ml). Response was linear from 2.5 to 80 μg/ml (r = 0.999); the recovery from spiked human serum was 76%. An external quality control was performed to compare this high-pressure liquid chromatographic method (H) with a standard microbiological assay (M); no significant deviation from slope = 1 and intercept = 0 was found by regression analysis (H = 1.03M – 0.45; n = 15).

Teicoplanin is a new glycopeptide antibiotic produced by Actinoplanes teichomyceticus (1, 10) which shows appreciable activity against staphylococci and streptococci (9, 13), corynebacteria (7), listeria (8), and clostridia (Y. Glupczynski, M. Labbe, F. Orokaert, and E. Yourassowsky, Letter, Eur. J. Clin. Microbiol. 3:50–51, 1984). It appears to inhibit cell wall biosynthesis in gram-positive organisms by interfering with peptidoglycan polymerization (12). Pharmacokinetic investigations showed a multiexponential concentration in blood-versus-time profile with elimination half-lives of 45 to 50 h (13), which is 6 to 10 times longer than that of vancomycin. In a recent review, Williams and Grünberg (14) reported on the in vitro and in vivo activity, pharmacology, and toxicology of teicoplanin. They recommended that levels in serum be monitored until the safety of this potentially valuable antibiotic is assured. Furthermore, they recommended additional studies to examine penetration of teicoplanin into various body fluids and to assess its therapeutic efficacy. Toxicity must be avoided in ongoing clinical trials by reasonable dosage recommendations formulated on the basis of concentrations in serum. Consequently, an accurate, sensitive, and specific assay for the determination of teicoplanin is needed.

The development of a high-pressure liquid chromatographic (HPLC) assay is difficult, since teicoplanin is a mixture of several related substances. The structures of its main components, A2-1, A2-2, A2-3, A2-4, and A2-5, and of the pseudoglycones, A3-1 and A3-2, have recently been elucidated (2, 3, 6). Several minor constituents, designated A2-1a, A2-1b, A2-1c, A2-1d, A2-1e, A2-3a, and A2-5a, were also detectable by use of a gradient HPLC method (A. Cometti, E. Riva, G. G. Gallo, and L. F. Zerilli, Clinical analytical methods brochure, 1984, Lepetit Research Center, Milan). The simplicity and speed of isotropic chromatography would be preferable, but previous nongradient experiments using UV or electrochemical detection were unsatisfactory with regard to resolution and detectability. Large sample volumes and tedious extraction steps were required due to poor UV absorbance of teicoplanin. Amperometric detection suffered from adsorption problems, which quickly passivated the surface of the electrode. Finally, rather different capacity factors of the individual components appeared to prohibit isocratic elution. However, these compounds all possess a primary amino group accessible to derivatization. We expected that their derivatives should exhibit an increased hydrophobicity and less-pronounced differences in polarity between each other. Indeed, an acceptable isotropic chromatography of this complex mixture became possible by derivatization before separation. A marked improvement of sensitivity was achieved simultaneously by use of fluorescence detection after precolumn reaction with fluorescamine.

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MATERIALS AND METHODS

Reagents. Teicoplanin sodium salt (lot 83HPO60; potency, 894 mg/g), obtained from Gruppo Lepetit S.p.A., Milan, Italy, was used as primary standard material. Analytical-grade n-butanol and fluorescamine (Fluka AG, Buchs, Switzerland), HPLC-grade methanol, acetonitrile (Rathburn Ltd., Wakeburn, Scotland), ion-pairing concentrate Pic-A (Waters Associates, Inc., Milford, Mass.), and double-distilled water were used.

Apparatus. The HPLC system consisted of a model 6000-A pump, a Wisp 710-B automatic sample processor, a model 840 data and control station (all from Waters Associates, Inc.), a model ERC-3310 in-line degasser (Erma Ltd., Tokyo, Japan), and a model 650-10-LC fluorescence spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.) operated at 390- and 490-nm excitation and emission wavelengths, respectively.

Chromatographic conditions. HPLC was performed at ambient temperature by using a reverse-phase Nova-Pak C18 column (15 cm by 3.9 mm; particle size, 5 μm [Waters Associates, Inc.]) and a flow rate of 1.3 ml/min. The mobile phase was a 10:10:1 (vol/vol) mixture of aqueous tetrabutylammonium phosphate solution (0.01 M; pH 7.5), methanol, and n-butanol.

Standards and specimens. A fresh solution of teicoplanin, corrected for drug potency, was prepared daily at a concentration of 1 mg/ml in distilled water. Working standards were freshly prepared by diluting this aqueous solution with...
antimicrobial-agent-free pooled human serum. A single 50-μg/ml standard was used for calibration, and final concentrations of 2.5, 5, 10, 20, 40, and 80 μg/ml were used for assessment of the assay performance (linearity, sensitivity, reproducibility, stability, and recovery). Twenty serum specimens, from patients receiving teicoplanin, were obtained from an external laboratory and kept frozen at −20°C until analyzed by HPLC. Bioassay results were obtained afterward for 15 of these quality control samples.

Sample preparation. Serum (100 μl) was thoroughly mixed with 200 μl of acetonitrile to precipitate the proteins and was centrifuged for 5 min at 5,000 rpm (2,000 × g). A portion of the supernatant (100 μl) was then diluted with 300 μl of borate buffer (0.2 M; pH 11.1), derivatized by the addition of 100 μl of fluorescamine (0.1% in acetonitrile), and stirred vigorously. Serum samples from patients and spiked serum standards were prepared in an identical manner and analyzed within 24 h after preparation. Injection volumes were usually 10 μl; for concentrations below 5 μg/ml, analysis was repeated with 20 μl.

Quantitation. Concentrations were calculated by comparison of the total peak area of six components with those of serum standards. An internal standard was not used, since no suitable substance was found. After four or less unknown specimens were analyzed, a standard sample was analyzed. Concentrations are expressed as the total amount of active teicoplanin components per volume. Quantitation of individual compounds was prohibited by lack of pure standard materials. Therefore, each component was presumed to produce an equal relative detector response.

RESULTS

Typical chromatograms of spiked and blank serum samples are shown in Fig. 1. The retention times were 10.2, 13.7, 15.7, 17.9, 23.2, and 25.4 min for active teicoplanin components A2-1, A2-2, A2-3, A2-3a, A2-4, and A2-5, respectively, with <1% variation over 24 h (n = 20). The undervaritized components previously have been separated by use of a semipreparative gradient chromatography adapted from the method of Borghi and co-workers (4). Partially purified compounds were derivatized and analyzed separately to permit unambiguous identification of the observed peaks (B. Joos and R. Luthy, submitted for publication).

A calibration curve, constructed by plotting peak area against concentrations of serum standards containing graded amounts of teicoplanin, showed a linear response from 2.5 to 80 μg/ml, with a correlation coefficient of 0.999. Recovery was 76% from spiked human serum (2.5 to 80 μg/ml) compared with a similar standard curve resulting from teicoplanin solutions prepared in water. The limit of detection was approximately 0.5 μg/ml. For six replicate measurements each made at 40, 20, 10, and 5 μg/ml, the variability (coefficient of variation of total area) was 1.5, 2.4, 1.5, 4.1, and 8.5%, respectively. No loss of fluorescence was observed in prepared samples stored for >24 h at room temperature.

The accuracy of the method was tested by comparing the HPLC results for coded serum samples, which ranged in concentration from 5 to 25 μg/ml, with the results obtained from a microbiological assay. Quality control samples were analyzed in an external laboratory (Gruppo Leptit S.p.A., Milan, Italy) with Bacillus subtilis as the test organism (5). The correlation between the two methods is shown in Fig. 2. Regression analysis was performed by the procedure of Passing and Bablok (11). The slope of the regression line was 1.030 (95% confidence interval from 0.900 to 1.130), and the intercept was −0.445 (confidence limits, −1.865 and 0.840).

The specificity of the assay was evaluated by analyzing aqueous solutions of various drugs at high concentrations. Over 50 antimicrobial agents were tested and shown not to interfere with this HPLC assay at levels exceeding their usual therapeutic range. These included six aminoglycosides, tested at >100 μg/ml (amikacin, gentamicin, neomycin, netilmicin, streptomycin, and tobramycin); 11 penicillins, tested at >300 μg/ml (amoxicillin, ampicillin, azlocillin, carbencillin, cloxacillin, flucloxacillin, mezlocillin, penicillin G, penicillin V, piperacillin, and ticarcillin); 13 cephalosporins, tested at >300 μg/ml (cefamandole, cefazolin, cefoperazone, cefotaxime, cefotiam, cefoxitin, cefsulodin, ceftazidime, ceftriaxone, cefuroxime, cefalothin, and moxalactam); four other beta-lactams, tested at >300 μg/ml (aztreonam, clavulanic acid, imipenem, and sulbactam); two glycopeptides, tested at >300 μg/ml (ristocetin and vancomycin); seven quinolones, tested at >100 μg/ml (cinoxacin, ciprofloxacin, enoxacin, norfloxacin, ofloxacin, pefloxacin, and pipemidic acid); and nine other antimicrobial agents, tested at >300 μg/ml (chloramphenicol, clindamycin, erythromycin, miconazole, ornidazole, rifampin, sulfamethoxazole, thiamphenicol, and trimethoprim). Interfering peaks found in one sample of human urine prohibited quantitation of the components A2-1 and

FIG. 1. HPLC chromatogram of teicoplanin in human serum (40 μg/ml, left) and of a blank serum control (right). A2-1, A2-2, A2-3, A2-3a, A2-4, and A2-5 denote active components of the teicoplanin complex.

FIG. 2. Comparison of teicoplanin concentrations in 15 serum samples from patients measured by bioassay and HPLC (regression line: y = 1.03x − 0.45).
A2-3a, at least at low concentrations. Nevertheless, determination of the major compounds of teicoplanin in urine appears feasible.

The flexibility of this method was examined by testing some potentially useful modifications. The speed of the assay could be increased by higher contents of butanol or acetonitrile in the mobile phase. No significant loss of resolution was observed after addition of approximately 0.5% more butanol, which decreased the run time by 50%. Moreover, the limit of detection could be decreased many times by injecting larger volumes. A serum sample from a patient containing approximately 3.6 μg of teicoplanin per ml was tested at seven different injection volumes (10, 20, 40, 60, 80, 100, and 150 μl). No significant distortion of the chromatograms was observed, and the response was perfectly linear.

DISCUSSION

The reversed-phase HPLC method described above is accurate, reproducible, specific, and highly sensitive. Sample preparation is simple, and precolumn derivatization with fluorescamine facilitates isocratic separation and determination of teicoplanin components A2-1, A2-2, A2-3, A2-3a, A2-4, and A2-5 in human serum. The classification of these different components of the teicoplanin complex is in agreement with the nomenclature of other investigators (data not shown).

Only small sample volumes (100 μl) are required, and the limit of detectability (0.5 μg/ml) is well below the usual therapeutic concentrations (5 to 50 μg/ml). By measuring fluorescence, absolute amounts of teicoplanin above approximately 670 pg are detectable. This represents an improvement in sensitivity by a factor of 100 to 1,000 compared with UV absorbance. Among the drawbacks of the assay are low recovery and lack of an internal standard. The reduced recovery is attributable to the influence of serum proteins or to incomplete reaction with the derivatizing agent. Therefore, serum standards must be used for calibration. Special attention must be paid to absolute injection volumes, since an internal standard was not included.

Nevertheless, this method has the potential for several helpful modifications to meet particular requirements with regard to speed, sensitivity, simplicity, and specimens. A new evaluation of the assay performance will be required, however, for such alterations. (i) The duration of analysis, under the conditions described, was 30 min per sample. When a higher rate is desirable for routine use, retention times may be decreased easily by a modification of the mobile phase. (ii) The assay was designed to measure therapeutic concentrations ranging from 5 to 50 μg/ml. When injection volumes of 20 μl are used, the lower limit of detectability is 0.5 μg/ml for the main component A2-2; whereas the minor components are difficult to quantitate at total teicoplanin concentrations below 5 μg/ml. Consequently, with injection volumes of 150 μl, the detection limit will be at approximately 0.07 μg/ml. Measurement of even lower concentrations would be possible by using alternate procedures for sample preparation, e.g., by enrichment of samples instead of a 15-fold dilution. (iii) A simplification of the assay could be achieved if only one component is determined. But a par pro toto analysis of teicoplanin A2-2 will be correct only if the relative composition of the teicoplanin complex remains constant in vivo. (iv) Finally, the utility of this assay could be expanded by testing its applicability to urine, cerebrospinal fluid, or tissue specimens.

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LITERATURE CITED