# IDENTIFICATION OF FLUORESCENT GLYCOPEPTIDE DERIVATIVES BY TWO CONSECUTIVE HIGH PRESSURE LIQUID CHROMATOGRAPHIC PROCEDURES

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Reversed phase high pressure liquid chromatography (HPLC) was used to separate individual components of the complex glycopeptide antibiotic teicoplanin in $\mu$g quantities with gradient elution. Each of eight different fractions was then subjected to a specific and highly sensitive HPLC method, which has been developed for the determination of teicoplanin concentrations in biological specimens. This analytical procedure includes pre-column derivatization with fluorescamine and isocratic elution. The fluorescent teicoplanin derivatives were identified by comparing their retention times in both HPLC procedures. Derivatization resulted in increased hydrophobicity and improved chromatographic separation, but the order of elution of the different compounds was not changed. The antimicrobial activity of the individual underivatized fractions correlated with their respective contents of total teicoplanin A2, whereas the pseudo-aglycone A3 appeared less active. Similar techniques have the potential to be applied to other complex glycopeptide antibiotics.

Teicoplanin was isolated from fermentation of *Actinoplanes teichomyceticus*[1,2], and is currently undergoing clinical trials because it possesses an appreciable biological activity and interesting pharmacological properties[3,4]. It belongs to the group of glycopeptide antibiotics, which are known to inhibit cell wall biosynthesis of Gram-positive organisms[5]. Monitoring of serum concentrations is routinely performed for vancomycin, the only glycopeptide in wide clinical use. Since it has not yet been shown that teicoplanin is less toxic, we have developed a HPLC assay for the determination of its concentration in biological specimens[6]. Teicoplanin has been shown previously to consist of several components. Gradient HPLC was performed by BORGHI and his co-workers[7], who used a solvent system of 10~40% acetonitrile in phosphate buffer and UV detection. They resolved the complex into the more polar compound teicoplanin A3 and five substances of lower polarity, designated teicoplanin A2. The structures of the closely related components A2-1, A2-2, A2-3, A2-4 and A2-5[8] and of the pseudo-aglycones A3-1 and A3-2[9,10] have been elucidated thereafter.

We used a fast and simple isocratic reversed phase HPLC method for the quantitative determination of different compounds of the teicoplanin A2 complex. Isocratic elution HPLC became possible by pre-column derivatization with fluorescamine, which reduced polarity differences between the individual components. Simultaneously the sensitivity was significantly improved by use of fluorescence detection. The performance of this assay was already described in detail elsewhere[6]. Briefly, response was linear and the reproducibility was satisfactory within the therapeutic concentration range, the limit of detection was approximately 670 pg of total teicoplanin, and the results correlated well with those obtained from a standard microbiological assay.

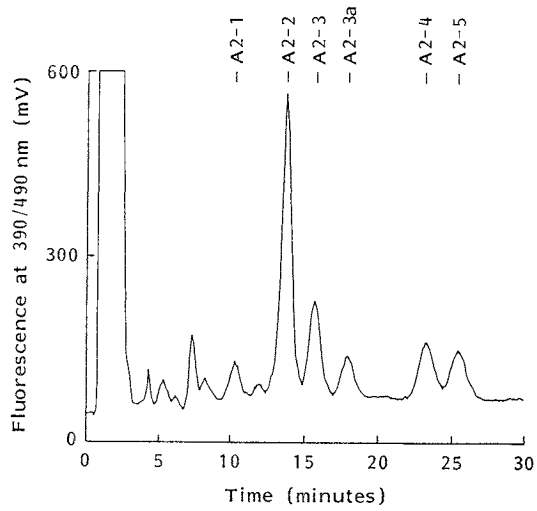
A typical chromatogram of a human serum sample containing teicoplanin is shown in Fig. 1.

Numerous components are eluted between approximately 5 and 25 minutes, whereas with blank serum samples no peaks were observed within this range. Coordination of peaks to specific teicoplanin components is anticipated here, since their order of elution cannot be predicted after derivatization.

The present work is concerned with the identification of the fluorescent derivatives measured by the analytical HPLC. This was accomplished by semi-preparative HPLC separation, followed by derivatization and analysis of the partially purified components of the teicoplanin complex. A comparison between both HPLC procedures is made and the antimicrobial activity of the individual fractions is tested.

Fig. 1. Representative isocratic HPLC chromatogram of teicoplanin in human serum (40 $\mu$g/ml).

A2-1, A2-2, A2-3, A2-4 and A2-5 denote fluorescent derivatives of the main active components of the complex.




## Materials and Methods

### Chemicals

Analytical grade BuOH and fluorescamine, HPLC grade MeOH and acetonitrile, double-distilled water and ion-pairing concentrate (Pic-A, Waters Assoc., Milford MA, U.S.A.) were used for the chromatographic procedures. Teicoplanin sodium salt (lot 83IP060, potency 894 mg/g) was obtained from Gruppo Lepetit SpA, Milan, Italy. The microbiological tests were performed using cation-supplemented Mueller-Hinton Broth (CSMHB, 1 mM $Mg^{2+}$, 1.25 mM $Ca^{2+}$) and Mueller-Hinton Agar (MHA, Difco Lab., Detroit MI, U.S.A.) as Nutrient media.

### Apparatus

The isocratic 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 Assoc.), a ERC-3310 inline degasser (Erma Ltd., Tokyo), and a model 650-10 LC fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk CT, U.S.A.). The gradient HPLC system included two pumps connected to a model 720 controller, a model U6K injector, a M480 UV detector and a M730 integrator (Waters Assoc.).

### Gradient HPLC

Semi-preparative separation was performed on a Resolve C18 column (150×3.9 mm, particle size 5 $\mu$m, Waters Assoc.) with a linear gradient from 10 to 40% acetonitrile in phosphate buffer during 20 minutes and a flow rate of 2.0 ml/minute. Crude teicoplanin solution (1 mg/ml in water) was injected and UV absorbance was monitored at 254 nm. Appropriate portions of the eluate were collected manually at the detector outlet, yielding eight different fractions (A~H). The corresponding eluates of four repeated injections (50~100 $\mu$l each) were pooled, resulting in total volumes of 2~4 ml per fraction.

### Isocratic HPLC

A reversed phase Nova-Pak C18 column (150×3.9 mm, particle size 5 $\mu$m, Waters Assoc.) was used for the analytical chromatography. The mobile phase, a 10:10:1 mixture of aq tetrabutylammoniumphosphate (0.01 M, pH 7.5), MeOH and BuOH was delivered at a flow rate of 1.3 ml/minute. Samples (0.1 ml) were diluted with 0.3 ml borate buffer (0.2 M, pH 11.1) and derivatized by addition of 0.1 ml fluorescamine (0.1% in acetonitrile) and subsequent vigorous stirring. Injection volumes of 20 $\mu$l were used and detection was at 390 and 490 nm excitation and emission wavelengths, respectively.

Microbiological Assay

A clinical isolate of *Staphylococcus aureus* was used for agar-well diffusion bioassay. Acetonitrile was evaporated from the chromatographically prepared samples in a stream of nitrogen and the original volumes were reconstituted with buffer. The test organism was grown overnight in CSMHB and added into MHA to a final inoculum of approximately $4\times10^4$ cfu per ml. The separated fractions and teicoplanin standards ranging in concentrations from 10 to 40 $\mu$g/ml were tested in duplicate. Wells (7 mm in diameter) were filled with 100 $\mu$l each, and the plates were incubated for 18 hours at 37°C.

## Results and Discussion

The semi-preparative gradient HPLC separation of underivatized teicoplanin is shown in the upper part of Fig. 2. The chromatogram is nearly identical with that obtained earlier by BORGHI *et al.*[7] in a similar solvent system. Resolution is inferior, probably since higher absolute amounts of teicoplanin were injected, and an internal reference was not used. Otherwise no relevant differences could be found. The six most abundant components (A and D~H) are therefore identical with teicoplanin A3-1, A2-1, A2-2, A2-3, A2-4 and A2-5, respectively, with decreasing polarity. Appropriate fractions of the eluate containing these compounds were collected, as well as the less important constituents designated A2-1b (B) and A2-1c (C). Several minor peaks between A and B including A3-2 and A2-1a were collected also, but their amounts were insufficient for further characterization. A total quantity of approximately 0.3 mg teicoplanin was subjected to several successive gradient HPLC separations and each component was eluted in several ml of the mobile phase. Consequently, the individual fractions were rather diluted and overlapping peaks, as expected, were only incompletely resolved.

The results of analytical HPLC of the derivatized fractions is illustrated in Fig. 3. The pseudo-aglycone A3-1 (fraction A) was not retained under the conditions of the isocratic HPLC. The minor constituents A2-1b and A2-1c (fractions B and C), teicoplanin A2-1 (fraction

Fig. 2. Gradient elution HPLC separation of the original complex (upper) and isocratic HPLC following pre-column derivatization (lower).

Aqueous solutions of teicoplanin were used. Connected peaks belong together.

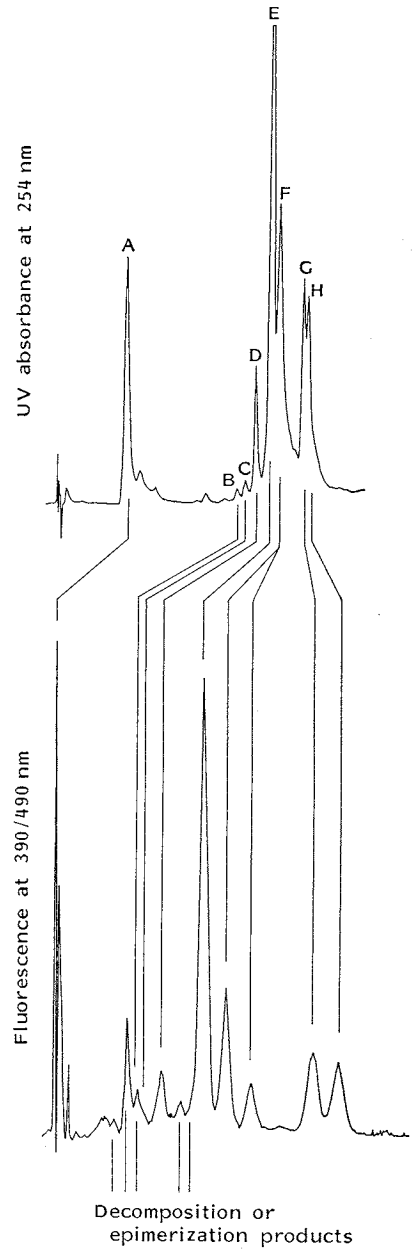
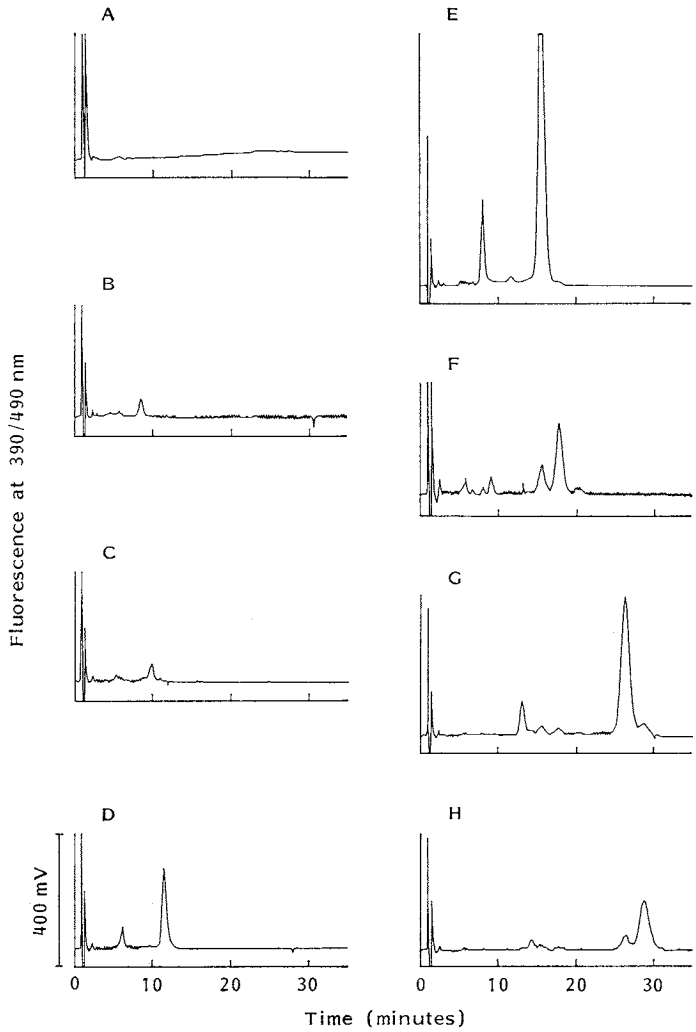


Fig. 3. Isocratic HPLC analysis of derivatized fractions A~H.




D), and the main component A2-2 (fraction E) were obtained in good chromatographic purity. Teicoplanin A2-3 (fraction F) still contained some teicoplanin A2-2 and was accompanied by an additional compound designated A2-3a. Teicoplanin A2-4 (fraction G) and teicoplanin A2-5 (fraction H) contained small impurities of each other.

It should be emphasized here, that these HPLC analyses of the individual fractions were performed after derivatization with fluorescamine. Besides the main derivatives, small peaks arising from at least five more polar substances could be observed in the chromatograms of fractions D~H. These are considered to be either decomposition or epimerization products originating under the alkaline reaction conditions during the derivatization procedure. The area of these five peaks account for 12~15% each, of the main compounds A2-1, A2-2, A2-3, A2-4 and A2-5, respectively. Moreover, these additional peaks are detectable also in the HPLC analysis of the derivatized teicoplanin complex in a pattern of relative distribution similar to that of the main components.

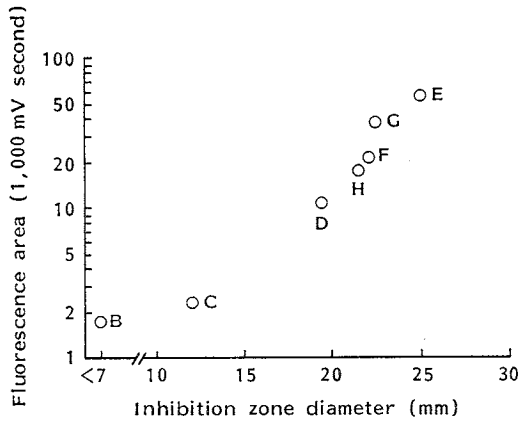
The lower part of Fig. 2 shows the isocratic HPLC chromatogram of a diluted aqueous solution

of the original complex. The different teicoplanin derivatives were recognized by their retention times, which were equal to those observed with the separate fractions (Fig. 3). Comparison with the gradient HPLC separation (Fig. 2, top) permitted their identification and demonstrated that the elution order of the teicoplanin components in reversed phase chromatography was not changed by derivatization with fluorescamine.

The antimicrobial activities of the particular components were estimated by agar diffusion. The area of the major peaks were taken as a measure for the relative amounts of teicoplanin present in the individual fractions and compared with their inhibition zone diameters (Fig. 4). The logarithmic correlation between zone diameters and fluorescence area demonstrated approximately equal specific activities. Comparative diameters of 22.5, 24.0 and 26.5 mm were obtained with buffered standard solutions containing 10, 20 and 40 $\mu$g/ml, respectively, of the teicoplanin complex. The pseudo-aglycone A3-1 (fraction A) could not be quantitated by isocratic HPLC, since it was not retained. The diameter of the inhibition zone for fraction A was only 19 mm, however, which was lower than that of fraction D (A2-1). But since A3-1 appeared to be much more abundant than A2-1, it was considered to be comparatively less active.

Fig. 4. Antimicrobial activity of individual fractions B~H.

Diameter of inhibition zone vs. relative amount of different teicoplanin A2 components.




In conclusion, the most active components of the teicoplanin complex were partially purified by use of gradient HPLC. Fractions of the eluate were taken directly for derivatization and isocratic elution HPLC analysis. Derivatization increased the hydrophobicity and facilitated chromatographic separation of individual compounds. Measuring fluorescence allowed the quantitative determination of even picogram amounts, whereas UV detection was less sensitive. Fluorescence detection may be desirable for the quantitative HPLC determination of other complex glycopeptide antibiotics as well. The high sensitivity and an improved chromatographic pattern after pre-column derivatization combined with the simplicity of the above-described identification procedure could be advantageous in this respect. Comparable gradient HPLC separations of numerous glycopeptides have been reported recently[11]. Their approximate order of elution in a similar solvent system and hence their increasing hydrophobicity was; ristocetin, LL-AM374, A-35512B, actinoidin, avoparcin, vancomycin, actaplanin, OA-7653, A-477, teicoplanin and aridicin. With the exception of ristocetin and vancomycin, these antibiotics are mixtures of several related substances and most of them contain a primary amino group accessible for derivatization with fluorescamine. Their analysis by using the same technique should be feasible with a few modifications.

Acknowledgments

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