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Quantitative determination of tautomeric FK506 by reversed-phase liquid chromatography

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Abstract

The quantitative determination of FK506, an immunosuppresant for organ transplants, was studied by using reversed-phase high performance liquid chromatography. There were three peaks corresponding to FK506 and its tautomeric compounds on the chromatogram obtained from aqueous solution. Interconversion among these compounds due to epimerization occurs and then reaches an equilibrium in aqueous solution. An increase in the water content in water–solvent solutions caused by the peak areas of FK506 and Tautomer I to decrease and that of Tautomer II to increase. For quantitative analyses of aqueous solutions this creates a problem because the composition of the mixture at equilibrium varies with the water content. A simple method, using a solution of polyoxyethylene lauryl alcohol ether (Brij-35) as a diluent, has been developed to provide a constant equilibrium. By diluting the sample with the Brij-35 diluent, it is possible to quantify FK506 in aqueous solutions. The reliability of the proposed method was confirmed with samples extracted from fermentation broth.

Keywords: FK506; Tautomerism; Equilibrium; Fermentation; Liquid chromatography

1. Introduction

Tacrolimus (FK506), a 25C, N,O-ring macrolide antibiotic isolated from *Streptomyces tsukubaensis* [1–3], is a potent immunosuppressant used in graft therapy [4,5]. FK506 has a unique structure (Fig. 1) and exhibits some interesting physico-chemical properties [6]. Two kinds of conformational heterogeneity of FK506 have been documented by nuclear magnetic resonance-(NMR) studies [7,8]. First, cis–trans conformational isomerization involving restricted rotation of the amide bond in the pipecolic moiety has been found [7], the isomerization being observed by the reversed-phase high performance liquid chromatography (HPLC) at a low column temperature [7,9] in a similar manner to that employed in the analysis of peptides containing proline residues [10]. FK506 exists in the cis conformation in the solid state [11]. Second, Namiki et al. [8] reported that in aqueous solution, FK506 epimerizes to an intermedite Tautomer I (cis) which is converted into Tautomer II (cis–trans) to

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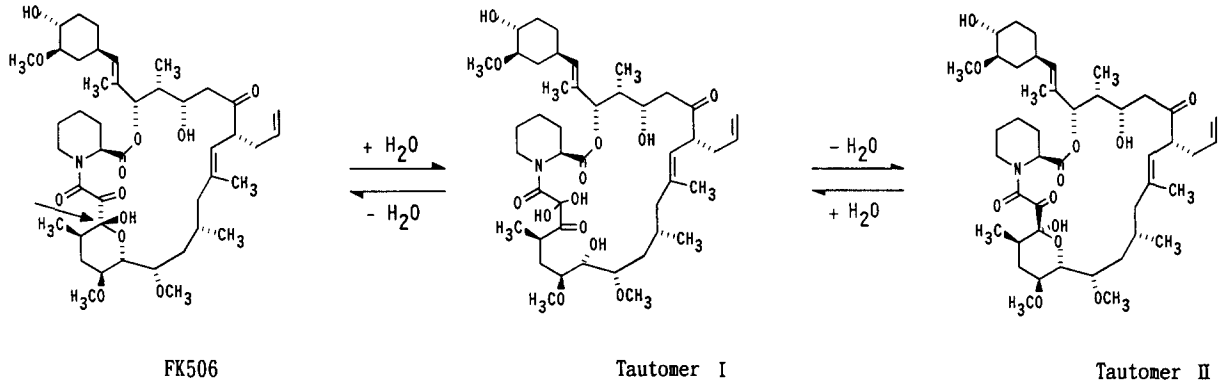

Fig. 1. Structure of FK506, Tautomer I and Tautomer II. The arrow in the FK506 structure indicates the centre of epimerization.

reach an equilibrium containing the three forms. The immunosuppressive activity was found to be associated with the cis–trans conversion, through binding to FK506 binding protein (FKBP) [12,13].

A quantitative chromatographic method for in-process monitoring of bulk drug substance in FK506 production was required to control processes, since the yield control is significant as a means of ensuring steady production with high quality.

Typical samples examined initially were fermentation samples containing water, solvents, a variety of unknown proteins, salts, etc., and by-products that can seriously hinder the accurate determination of the components of interest. Moreover, it was considered that multiple peaks resulting from the tautomerism would make quantitative analysis more complex. As will be demonstrated, the equilibrium is dependent on water content in aqueous solution. When there is a difference in water content between a sample and a standard solution, general quantitative analytical methods such as the external standard method are unsuitable. There is a need for a reliable methodology for the accurate quantitative analysis of tautomeric FK506. The goal of this work was to develop a chromatographic method suitable for in-process monitoring. Since many of the samples involved contain various amounts of water, an investigation was started to elucidate the nature of FK506 in aqueous solution. For this purpose reversed-phase HPLC was employed because not only does it offer several advantages such as high resolution and sensitivity, but also it could be applied to aqueous solutions without tedious sample preparation. Consequently, we could observe changes in equilibrium in aqueous solution. Reversed-phase HPLC profiles at various column temperatures were obtained for kinetic study of the cis–trans interconversion by Nishikawa et al. [14], but they did not mention the chromatographic behaviour of tautomeric peaks.

This paper reports the investigation of the tautomerism in aqueous solution using a simple quantitative reversed-phase HPLC method. The method was evaluated for linearity, accuracy, precision, and specificity.

2. Experimental

2.1. Chemicals and reagents

FK506 ($C_{44}H_{69}N_{12} \cdot H_2O$) and immunomycin (FR900520) [15] were prepared at Fujisawa Pharmaceutical (Aichi, Japan). Polyoxyethylene lauryl alcohol ether (Brij-35) was a specially prepared reagent for automated amino acid analysis (Nakalai Tesque, Kyoto, Japan). Water was purified by using a Milli-Q system (Millipore, Bedford, MA, USA). All other solvents and reagents were of analytical or HPLC grade and were commercially available. To prepare the Brij-35 diluent, 50 g of Brij-35, 12.2 g of sodium dihy-

drogenphosphate dihydrate and 43.8 g of disodium hydrogenphoshate dodecahydrate were dissolved on a water-bath. After cooling, the solution was made up of 1000 ml by adding water and filtered through a 0.45 μm in membrane filter.

2.2. Chromatography

The HPLC system consisted of an 880-PU pump (Jasco, Hachioji, Japan), a KMT-120A autosampler (Kyowaseimitu, Mitaka, Japan) a model 875 UV detector (Jasco) operated at 215 nm, combined with a C-R4A chromatographic data processor (Shimazu, Kyoto, Japan). A 4.0 mm i.d. × 250 mm LiChrospher RP-18 column (LiChroCART, 5 μm; Cica-Merck, Tokyo, Japan) was immersed in a constant-temperature bath at 50°C. The flow rate of the mobile phase was 2.0 ml min^{-1}. Acetonitrile–water–phosphoric acid (600:400:1, v/v/v) was used as the mobile phase. The role of the phosphoric acid was to speed up the elution of impurities derived from the fermentation. The injection volume was 20 μl.

2.3. Preparation of samples and standard solution

FK506 was dissolved in pure solvents because of its low solubility in aqueous solutions (*n*-octanol–water partition coefficient >1000 [6]). For the equilibrium study, the samples were prepared by adding equal volume of water to a freshly prepared acetone solution of FK506, except where mentioned otherwise. In the Brij-35 study, the acetone solution was diluted with the Brij-35 diluent and then acetone was added to make test solutions of the desired acetone concentrations in which the FK506 content was constant. The solutions were allowed to stand at ambient temperature for 1 h and then injected into the HPLC system.

The fermentation extract was prepared by adding an equal volume of acetone to the fermentation broth. After thorough mixing the suspension was allowed to stand for 1 h and then filtered through filter-paper. For HPLC analysis, the sample was prepared by diluting with the Brij-35 diluent to give solvent concentrations in the range 0.5–16%. After not less than 15 min, the sample was injected. Stock standard solutions were prepared in acetone. Appropriate dilutions of the solutions were carried out in the same way as descsribed above prior to use.

For the calibration study, a 1 mg ml^{-1} acetone solution of FK506 was diluted with the Brij-35 diluent at 100, 80, 60, 40, 20, 10 and 5 μg ml^{-1} to give acetone concentrations of 10, 8, 6, 4, 2, 1 and 0.5%, respectively. For the reproducibility study, test solutions made up to solvent–water (40:60, v/v) of acetone, ethanol and acetone, ethanol and acetonitrile were analysed. A recovery study was carried out by adding FK506 in the concentration range 5–20 μg ml^{-1} intervls to the extract containing FK506 at 100.2 μg ml^{-1}.

2.4. Data analysis

To evaluate the equilibrium of the tautomerism, each peak area of Tautomers I and II and FK506 was calculated as follows. For example,

$$\text{peak area (\%) of Tautomer I} = \frac{A_{\mathrm{TI}} \times 100}{A_{\mathrm{TI}} + A_{\mathrm{TII}} + A_{\mathrm{FK506}}}$$

where A_{TI}, A_{TII} and A_{FK506} are the peak areas of the Tautomers I and II and FK506, respectively. The other peak areas were calculated in the same way.

To obtain the calibration graph and determine recovery, the product–moment correlation coefficient and regression line were calculated.

3. Results and discussion

3.1. Preliminary investigation

Several mobile phases and column temperatures were evaluated for their applicability to measure FK506 in aqueous solutions. A high column temperature (50°C) was necessary to gain good peak shape and separations for chromatography. There were three peaks corresponding to FK506 and Tautomers I and II on the typical chromatogram obtained from an aqueous solution (Fig. 2), in which separation of the cis–trans isomers was not

observed. Since the interconversion rate between the two forms is rapid at higher temperatures, a fused peak is obtained [14]. In this study, separation of the cis–trans isomers was not necessary. During this investigation, it was observed that each area of the three peaks varied between the solutions in which the FK506 content was constant. In chromatography based on measuring peak areas of bands, when the peak areas vary like this quantitative determination is problematic. In the mechanism of formation of the tautomers (Fig. 1) [9], the water molecules play an important role; FK506 and Tautomer II are formed by removing a water molecule from Tautomer I, which is reversibly formed by the addition of water. Therefore, it could be assumed that the changes in peak areas were due to the tautomeric equilibrium that was seriously affected by the water content in the solutions. The first investigations performed was to survey the tautomerism in aqueous solutions. Then, three possible factors affecting the reactivity in aqueous solution were examined: the water content, the pH and the temperature.

3.2. Studies on equilibrium

Effect of water

To ascertain whether the water could acount for the changes in peak areas, solutions prepared in the range acetone–water (30:70 to 90:10, v/v) with a constant amount of FK506 were left overnight and injected into the HPLC system. Because of the low solubility in aqueous solution, the water content could not be increased to more than the 70% (v/v). In Fig. 3, the peak areas of both FK506 and Tautomer II decreased with increasing water volume, whereas that of Tautomer I increased. The same results were obtained with other polar solvents such as methanol, ethanol, acetonitrile and tetrahydrofuran. Depending on the water content, FK506 in aqueous solution was in equilibrium as had been assumed.

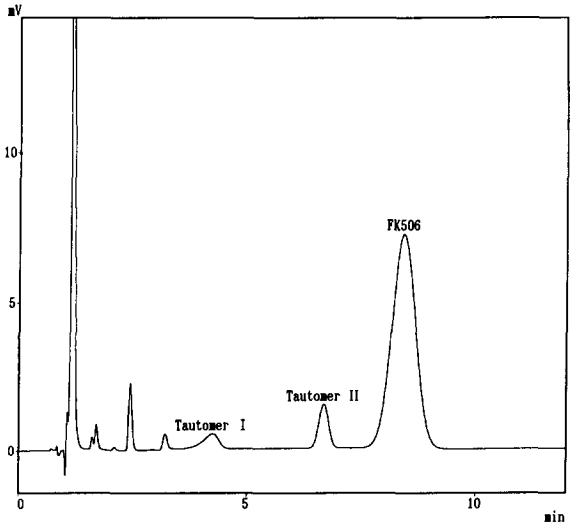

Fig. 2. Typical chromatogram of FK506 in acetonitrile–water solution (50–50, v/v). For chromatographic conditions, see Experimental section.

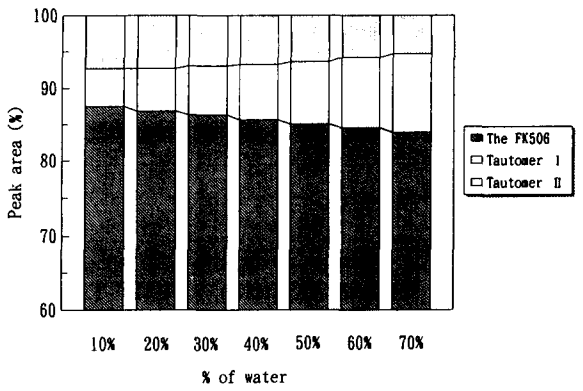

Fig. 3. Effect of water concentration on tautomeric equilibrium. A 100 $\mu g\,ml^{-1}$ solution of FK506 in acetone–water was left overnight at ambient temperature and then analysed. ●, pH 3; ◇, pH 5.3; ▲, pH 7.3.

Effect of pH

To examine effect of pH, the solutions of various pH prepared by adding McIlvaine buffer to the acetone solution were analysed as a function of time. Fig. 4 shows the kinetic behaviour of the equilibrium in aqueous solutions. Although the rate of attaining equilibrium at neutral pH was higher than that at acidic pH, the peak areas eventually became constant under acidic to neutral pH conditions.

Effect of temperature

The effect of temperature was examined in the same way as desribed above using acetone–water

(50:50, v/v) at 3, 26 and 50°C. The rate accelerated with increase in temperature, as expected. Each peak area eventually became constant, as in the pH study, except at 3°C, where equilibrium could not be attained during the experimental period. The times needed to attain common equilibrium were as follows: less than 40 min at 50°C, ca. 350 min at 26°C and more than 500 min at 3°C.

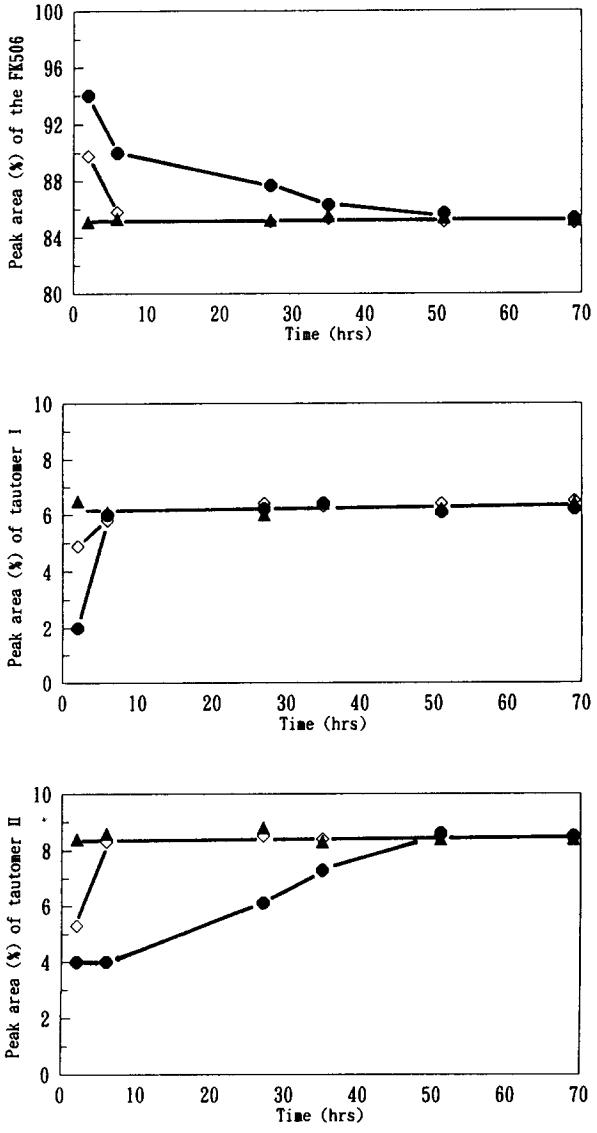

Fig. 4. Effect of pH on equilibrium. A 100 μg ml^{-1} solution of FK506 in acetone–buffer (50–50, v/v; pH*3.0 (●), 5.3 (◇) and 7.3(▲)) was prepared and then analysed as a function of time.

3.3. Preparation for quantitative analysis

The possibility of developing a quantitative reversed-phase HPLC method was investigated. It should be noted that samples to be studied contained various amounts of water, which affect the changes in equilibrium. Basic quantitative analytical methods, such as the internal and external standard methods, can be employed in most cases. However, there was inconsistency in the equilibria between the samples and the standard solutions as calibrators, which adversely affects the performance of the quantitative methods. Conversely, if the equilibria in all samples including the standards were constant, the assay results could be calculated with an arbitrary peak area in a set of three peaks for each sample to be chromatographed. Therefore, for successful quantitative analysis the equilibrium must be controlled. One possible method ensuring a constant equilibrium using a solution prepared with strict control of the water content was developed. However, this approach was unsuitable for the present samples as it required tedious and time-consuming sample preparation. Therefore, consideration was given to equilibria in solutions rich in water, which might be more stable to small changes in water content in the presence of sufficient water. It was found that Brij-35, a typical non-ionic surfactant having low ultraviolet absorbance, could be used for increasing the solubility in water. Brij-35 was applied as a diluent and then the equilibrium in the diluted mixture was examined. The Brij-35 diluent needed to contain 5% (w/v) Brij-35 to prevent insolubilization of FK506 in the mixture. Also, buffering pH7.0 with phosphate rapidly establishes a new equilibrium. As the actual samples contained various species and unknown amounts of solvents, the most important role of the diluent was to obtain a constant equilibrium. Hence it was necessary to estimate the equilibria of the mixtures to be assayed.

Equilibrium in the Brij-35 diluent

The Brij-35 diluent had no effect on the chromatographic patterns. To estimate the equilibria

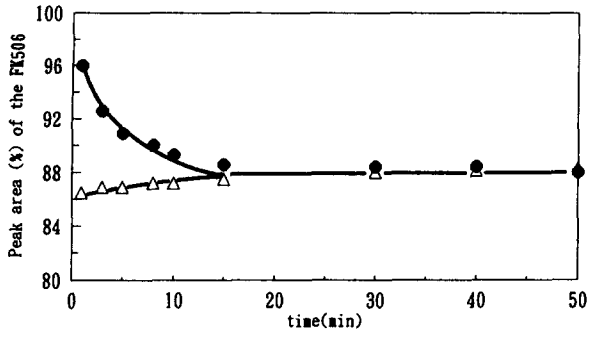

Fig. 5. Time to allow the establishment of equilibrium. Samples were prepared from acetone (●) or acetone–water (40:60, v/v) (△) and then analysed as a function of time.

of the mixtures, test solutions containing acetone in the concentration range 0.5–64% (v/v) were analysed. There was a similar tendency for the acetone content to the results for water–solvent described above. However, in the range 0.5–16%, the equilibriua seemed to be constant. In detail, although the peak areas of Tautomers I and II slightly increased and decreased, respectively, with decrease in the acetone content, that of FK506 remained constant throughout this range. The rate to attain the equilibrium was evaluated by analysing mixtures diluted tenfold from both solutions of acetone and acetone–water (40:60, v/v) at room temperature. Fig. 5 shows the shifts of the peak area with time. Having a constant equilibrium, both mixtures attain re-equilibrium in about 15 min.

To study the applicability of solvent solutions other than acetone, similar experiments were performed using ethanol and acetonitrile with acetone as the control. In each case the FK506 peak area was constant and remained so for more than 8 h. These results indicated that success of the quantification was ensured.

3.4. Quantitative analysis study

A typical chromatogram of the sample extracted from fermentation broth is shown in Fig. 6. Although a good separation of the FK506 peak from the polar residues was obtained, by-products which eluted earlier than FK506 disturbed the baseline separation between Tautomer II and FK506. By observing the tautomeric behaviour and the chromatographic separations, a determination method was developed and evaluated. As the presence of various amounts of impurities derived from the fermentation may interfere with the peak area measurements, this method employed an external standard procedure for measuring the FK506 peak height.

Linearity and precision

A calibration graph was obtained by analysing aqueous solutions which contained 0.5–10% acetone with FK506 in the concentration range 5–100 $\mu g\,ml^{-1}$. In spite of the wide difference in the acetone content, good linearity and a zero intercept were observed between the peak heights and corresponding FK506 concentrations. The regression equation was $y = 0 + 87.48x$ ($r^2 = 0.9999$), where y is the peak height and x is the FK506 concentration ($\mu g\,ml^{-1}$).

Next, intra-assay precision studies were carried out to evaluate the reproducibility of the method and the matrix effect on solvent species in the sample solutions. The results compared with those of acetone solutions were $98.0 \pm 0.7\%$ (mean $\pm$ SD, $n = 8$) for the ethanol solution and $99.6 \pm 0.3\%$ for the acetonitrile solution, based on an acetone solution value of $100 \pm 0.7\%$.

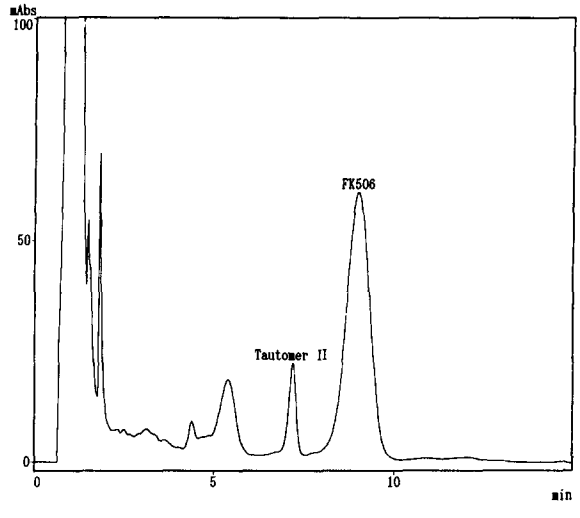

Fig. 6. Separation of the extract obtained from fermentation broth.

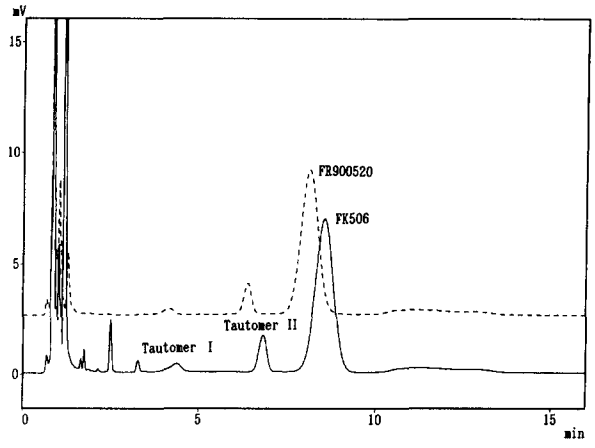

Fig. 7. Elution profiles of FK506 and FR900520. Peaks corresponding to Tautomers I and II are clearly shown in the FR900520 chromatogram.

Standard recovery

The equation of the regression line was $y = 100.1 \pm 0.970x$ ($r^2 = 0.9997$) where y is the determined amount ($\mu g\,ml^{-1}$) and x is the amount of FK506 added to the solutions ($\mu g\,ml^{-1}$). From this equation, the recovery was calculated statistically as 97.0% and the intercept nearly equalled the determined value for the original extract.

Interference of FR900520

FR900520, a by-product of FK506 fermentation, is eluted just before the FK506 peak, as shown in Fig. 7. Therefore, its peak must overlap with that of FK506, interfering with the measurement of FK506. To establish whether such an interference would occur, FK506 measurements were performed on fermentation extracts spiked with FR900520. A comparison of the results based on both peak area and peak height is shown in Fig. 8. The presence of FR900520 does not affect the measurement of the FK506 peak.

4. Conclusion

The tautomeric behaviour of FK506 in aqueous solution was studied using reversed-phase HPLC. It was shown that the tautomeric equilibrium between FK506 and Tautomers I and II is dependent on the water content in solvent–water solution. With increasing water content, the equilibrium tends to increase the Tautomer I level with decreasing FK506 and Tautomer II levels. Changes in water content bring the solution to a new equilibrium, and the rate of attaining the equilibrium is affected by temperature and pH of the solution. Therefore, the equilibria in solutions containing various amounts of water cannot be constant. An aqueous solution is in an equilibrium corresponding to its water content or changes to it.

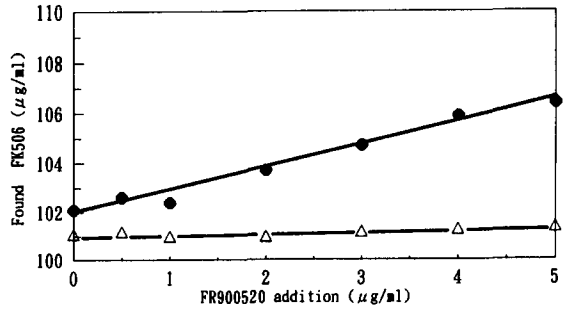

Fig. 8. Comparison of determination methods based on peak height (△) and peak area (●). Samples were prepared by spiking FR900520 into the fermentation broth extract.

The quantitative LC determination of FK506 was aided by controlling the equilibrium. As samaples diluted with the Brij-35 diluent are brought to a constant equilibrium, the determination can be easily performed with general procedures for HPLC analysis. For the analysis of samples extracted from fermentation broth, the external standard method based on the FK506 peak height was employed. This method was shown to be very reliable.

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