Comparison of High-performance Liquid Chromatography and Microbiological Assay for Determination of Josamycin in Rat Serum

by

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Abstract

Josamycin (JM) was given orally to 10 rats at a dose of 200 mg/kg. Serum samples were obtained at 60, 120, 180, and 240 minutes after oral administration and JM concentrations in serum were determined by both high-performance liquid chromatography (HPLC) and a microbiological assay. Comparison of the JM concentrations in serum measured with HPLC and microbiological assay by regression analysis yielded a markedly high constant of inclination (Y = 8.0719X - 0.2995, r = 0.9867, where Y is JM concentration obtained with microbiological assay and X is that obtained with HPLC; r is the regression coefficient). The difference indicated that most of the JM was metabolized and that the presence of antimicrobially active metabolites, deisovaleryl-JM being a main constituent which was also measured as JM, plays an important role in the microbiological assay. Thus, the microbiological assay might not be a suitable method for determination of the unchanged antibiotic if it is metabolized rapidly to produce antimicrobially active metabolites.

Introduction

Because of its ease of use and sufficient accuracy and sensitivity, a microbiological assay (agar diffusion method, such as paper disc method) has long been applied for the determination of antibiotics in body fluids and various organs in studies of the bioavailability, bioequivalence, distribution, and pharmacokinetics of antibiotics.

However, the result obtained from this method indicates the antimicrobial activity of the antimicrobially active substances as a whole, such as that of the given antibiotic and its active metabolites. Therefore, it is impossible to determine the true concentration of a given antibiotic if it is metabolized rapidly to form anti-
Josamycin (JM) is one such antibiotic which is metabolized rapidly to produce metabolites possessing antimicrobial activity\(^{11}\). OSONO et al.\(^{12}\) studied the metabolism of JM in man and animals including rats, dogs, and monkeys. In the case of rats, the main metabolite was deisovaleryl-JM, which accounted for 96.6\% of the metabolites in first 24-hour urine (Fig. 1). Since the metabolites of JM possess antimicrobial activity, the results obtained by microbiological assay do not indicate the true amount of unchanged JM.

The present investigation was therefore undertaken to clarify the relationship between HPLC and microbiological assay for determination of JM in the serum of rats after oral administration of the drug.

**Materials and Methods**

1) **Animals**

Wistar strain male rats, 5 weeks old, weighing 80–100 g, were purchased from Sankyo Lab. and reared in our animal center for 8 weeks. Rats were maintained on a commercial diet (Oriental MF, Oriental Yeast Co., Ltd.) and filtered tap water *ad libitum* at a room temperature of 23 ± 1\°C, a humidity of 60 ± 5\%, and a 12-hour lighting cycle. The body weight of each rat at the start of the experiment was approximately 350 g.

2) **Chemicals**

Josamycin (JM, Lot No. JSM-Y198, pot. 975 µg/mg) was a gift from Yamanouchi Pharmaceutical Co., Ltd. The other chemicals were purchased from Wako Pure Chemical Industries Ltd., except for where indicated.

3) **Experimental Procedure**

Ten rats were fasted for 24 hours and then administered JM at a dose of 200 mg/kg (10 mg of JM was dissolved in 1 ml of purified water with the addition of
2 N HCl) through a stomach sonde. Blood samples were obtained from the jugular vein 60, 120, 180 and 240 minutes after administration and the sera were harvested. For the microbiological assay, each serum sample was diluted to an appropriate concentration with 1% phosphate buffer, pH 6.0. For HPLC, 100 μl of serum and 200 μl of absolute methanol were mixed vigorously and then centrifuged to remove denatured proteins. The supernatant (50 μl) was injected into the HPLC system.

4) Microbiological Assay

JM concentration in serum was assayed using a thin-layer disc plate method employing Micrococcus luteus strain ATCC 9341 as the test organism. Bacto-Neo-mycin Assay Agar (Antibiotic Medium 11, Difco) was used as the assay medium. The minimum detectable concentration by this method was approximately 0.1 μg/ml of JM. Standard JM was dissolved in rat serum and diluted with the same serum to obtain final concentrations of 10, 7.5, 5, 2.5, 1, 0.5, 0.1, 0.05, and 0.01 μg/ml. All samples and standards were assayed in triplicate.

5) High-performance Liquid Chromatography (HPLC)

The HPLC system was composed of a Liquid Chromatograph Model 510 (Waters), a μBondapak C₁₈ (main column, Waters), a Huard column (pre-column, BAS), a TCM (Waters Temperature Control System), and a Lambda-Max Model 481 LC Spectrophotometer (Waters). The column temperature was 35°C. The mobile phase was 0.01 M sodium acetate-acetonitrile (54:46, v/v) and the flow rate was 2.0 ml/minute (pressure: approximately 211 kg/cm²[3, 4]). UV absorption at 230 nm with a sensitivity of 0.02 AU was drawn on a recorder. Peak area was calculated using the following equation:

\[
\text{Area (mm}^2\text{)} = (\text{Peak width at half-height}) \times (\text{Peak height})
\]

A standard concentration-area curve was obtained by mixing JM solution (100 μg/ml) and rat serum, and then the mixture was added to twice the volume of absolute methanol. After centrifugation (1,500 × g), the supernatant (50 μl) was injected into the HPLC system.

The minimum detectable concentration by this method was approximately 0.025 μg/ml.

Results

1) Microbiological Assay

The time-concentration curve of JM in rat serum after oral administration at a dose of 200 mg/kg is shown in Fig. 2. The JM levels in serum (mean±SD) at 60, 120, 180, and 240 minutes after oral administration were 13.54±3.49, 5.67±1.64, 2.94±0.96, and 3.49±0.78 μg/ml, respectively.

2) High-performance Liquid Chromatography (HPLC)

The standard concentration-area curve is shown in Fig. 3. The peak area was proportional to the amount of JM injected (r=0.9991, p<0.0001 for methanol treatment and r=0.9998, p<0.0001 without treatment). The yield after methanol treatment was calculated from the slope and was found to be 94.5%.

The time-concentration curve of JM in rat serum is shown in Fig. 4. The JM levels in serum (mean±SD) at 60, 120, 180, and 240 minutes after oral administration were 1.70±0.31, 0.65±0.20, 0.58±0.16, and 0.44±0.15 g/ml, respectively.
Fig. 2 Time-concentration curve of josamycin in rat serum after oral administration. The serum concentration was measured by microbiological assay.

Fig. 3 Standard concentration-area curve of josamycin measured by high-performance liquid chromatography (HPLC). Dotted line indicates non-methanol treatment ($r=0.9998$, $p<0.0001$) and solid line methanol treatment ($r=0.9991$, $p<0.0001$).
Discussion

HPLC analysis of JM in a biological sample is a rather troublesome task. RADER et al.\cite{3} developed a method of direct injection of plasma or homogenized blood cells using alternating special sample clean-up columns requiring a time-consuming complicated process. DUCCI and SCALORI\cite{4} used an extraction procedure for the preparation of injection with a relatively low yield (71-91%). The present method involving the addition of absolute methanol and the use of a small precolumn system was quite easy and gave a sufficiently high recovery (94.5%) and sensitivity (0.025 µg/ml). Thus, the present method is superior to those proposed previously.

Joos et al.\cite{5} reported a comparison of high-pressure liquid chromatography and bioassay for determination of ciprofloxacin in serum and urine and demonstrated that the comparison of the concentrations in serum by regression analysis yielded a slope which was not significantly different from 1.0 (p<0.001). However, in case of urine, the bioassay results were markedly higher than the high-pressure liquid chromatography values, thus indicating the presence of antimicrobially active metabolites in the urine. As demonstrated by OSONO et al.\cite{11}, JM was also transformed to give antimicrobially active metabolites, and thus it was quite reasonable that the microbiological assay gave a much higher serum level of JM than that given by HPLC.

The present data on the microbiological assay indicated that the peak was equal to or more than 13.54 µg/ml. OSONO et al.\cite{11} reported a peak concentration of 13.67 µg/ml (ranging from 10.9 to 18.0 µg/ml) 2 hours after oral administration of JM at a dose of 400 mg/kg in rats. KURIAKI et al.\cite{6} reported a peak concentration

![Fig. 4 Time-concentration curve of josamycin in rat serum after oral administration. The concentration was measured by high-performance liquid chromatography (HPLC).](image)
of 31.0 μg/ml 2 hours after oral administration at a dose of 100 mg/kg in dogs. Thus, the present results measured by microbiological assay were of a reasonably correct order. In contrast, the serum concentration of JM obtained by HPLC was much lower (1.70 g/ml at maximum) than those obtained by the microbiological assay. This strongly suggested that a relatively large amount (approximately 87%) of antimicrobiologically active metabolites existed in the serum.

The relationship between the JM levels obtained by HPLC and those by microbiological assay is shown in Fig. 5. The regression coefficient was 0.9867 (p<0.005), which indicated that the amount of JM was directly proportional to that of its antimicrobiologically active metabolites. Since deisovaleryl-JM is the main metabolite of JM in the rat[2], and since it also may possess antimicrobial activity, which has been indicated by TLC[1,2], deisovaleryl-JM might play an important role in the antimicrobial activity demonstrated by microbiological assay.

Thus, the microbiological assay might not be a suitable method for determination of unchanged antibiotic if it is metabolized rapidly to produce antimicrobiologically active metabolites.

**Conclusions**

JM levels in rat serum after oral administration were measured by both HPLC and by microbiological assay. The HPLC method for measurement of JM in serum was simplified in the present study without any loss of accuracy or sensitivity. The amount of unchanged JM in rat serum was approximately 13% and the
antimicrobially active residue was found to be deisovaleryl-JM.

Thus, the microbiological assay is not suitable for determination of unchanged true JM in rat serum.

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References