High Performance Liquid Chromatographic Determination of Clarithromycin in Lymphocytes Using a Post-column with Tris(2,2′-bipyridine) Ruthenium (III) Chemiluminescence Detection

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The concentration of antibiotics in cells must be determined for effective treatment of infectious diseases caused by obligate intracellular parasites, such as *Chlamydia trachomatis* and *Legionella pneumophila*. We confirmed the usefulness of high performance liquid chromatography (HPLC), which is already commonly used in medical facilities, for the measurement of antibiotics in cells. The measurement was carried out using a post-column with tris(2,2′-bipyridine) ruthenium (III) chemiluminescence detection. Clarithromycin (CAM) was used as the model antibiotic. The retention time of CAM on the column was 7.6 ± 0.4 min and the detection limit was 2.0×10^{-3} ng on column. The linearity of the calibration curve was guaranteed until 2.0 ng on column. This level of performance is comparable to that of liquid chromatography-tandem mass spectrometry. The system was able to monitor changes in the concentration of CAM in lymphocytes. These findings suggest that HPLC, a general-purpose, already widely used detection system, could also contribute to the development of effective individual antibiotic treatment regimens at a wide variety of medical facilities.

Key words —— liquid chromatograph, chemiluminescence, clarithromycin, lymphocyte

INTRODUCTION

About a decade ago, the medical establishment became aware of an increase in the number of patients with sexually transmitted disease and atypical pneumonia, and the pathogens involved were found to be obligate intracellular parasites, such as *Chlamydia trachomatis* and *Legionella pneumoniae*. These organisms parasitize lymphocytes, thereby evading the host's immune system and the effects of extracellular antibiotics.[1] Thus, knowledge of the ability of various antibiotics to be transferred into cells is essential when attempting to treat infections caused by bacteria capable of remaining alive inside cells. Accordingly, it is important to estimate the concentration of the antibiotic in the cell rather than in the blood stream when the appropriate dosing regimens are being determined.[2,3] To this end we need to establish a system to measure the concentration of antibiotics in cells.

Macrolide antibiotics are the drug group of choice to treat patients with chlamydial infections and legionella pneumonia.[4,5] Some authors have suggested analytical methods such as the bioassay method and liquid scintillation counter method to determine the concentration of antibiotics in cells.[6,7] However, the bioassay is a lengthy procedure and produces large measurement deviations, while the liquid scintillation counter method has the inherent difficulties associated with the use of radio isotopes. Liquid chromatography-tandem mass spectrometry (LC-MS-MS) has also been suggested for the quantitative analysis of macrolide antibiotics in plasma,[8,9] and has high sensitivity and produces highly accurate identification. However, the apparatus is expensive and complicated to maintain, thereby limiting its acquisition and use by a wide range of medical facilities. Thus, the development of a measurement procedure that can be used with pre-existing equipment already common in medical facilities, or inexpensive to acquire, is required, and to this end we examined liquid chromatogra-

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phy (LC). LC is widely used in many types of medical facilities for many analytical purposes, including therapeutic drug monitoring.

Studies have already shown that LC with a UV detector is not sufficiently sensitive to measure the concentration of macrolide antibiotics in plasma.[10, 11] Thus, in the present study, we examined whether LC using a tris(2,2′-bipyridine) ruthenium (III) chemiluminescence detection system would be suitable for the quantitative analysis of macrolide antibiotics in cells. The ruthenium complex $(Ru(bpy)_3)^{3+}$ has specific binding activity to the alicyclic tertiary amine structure found in macrolide antibiotics, and is currently used for chemiluminescence analysis.[10–15] Clarithromycin (CAM) was used in the present study as the representative macrolide antibiotic since it has been widely used for a long time in Japan.

MATERIALS AND METHODS

Reagents and Materials —— Tris(2,2′-bipyridine) dichlororuthenium(II) hexahydrate ($[Ru(bpy)_3Cl_2 \cdot 6H_2O]$), roxithromycin (ROX), RPMI-1640 medium, Hanks' balanced salt solution (HBSS), gentamicin (GM), vancomycin (VM) and amphotericin B (AMPH) of reagent grade were purchased from Sigma-Aldrich Co. (Milwaukee, WI, U.S.A.). Fetal bovine serum (FBS) and human AB serum were purchased from Invitrogen Co. (Carlsbad, CA, U.S.A.). CAM (biochemical reagent grade), other chemicals of analytical grade, and HPLC grade solvents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

The human T-lymphocyte cell line MOLT-4 was obtained from American Type Culture Collection (Manassas, VA, U.S.A.). Human lymphocytes were obtained by fractionation of blood drawn from volunteers after informed consent was obtained and the procedure approved by the Ethics Committee of Showa Pharmaceutical University.

Apparatus and Chromatographic Conditions —— The HPLC system consisted of two HPLC pumps (DP8020, Tosoh CO., Tokyo, Japan), a Model 7125 injector with a 20 μl loop (Reodyne, Inc., Cotati, CA, U.S.A.), a TSK-GEL ODS-100 V column (150 × 4.6 mm i.d., Tosoh) and a Model Comet-2000 electrogenerated chemiluminescence detector (Comet, Kawasaki, Japan) with a flow cell of 80 μl, a Multi Station LC-8020 unit (Tosoh) and a CO-8020 column oven set at 40°C (Tosoh). Thermostatic oven of Comet-2000 was also set at 40°C. Under controlled current electrolysis, the current was maintained at 100 μA. The reagent solution was dissolved to 0.25 mM of $Ru(bpy)_3Cl_2$ in 10 mM sulfuric acid. The mobile phase was a mixture of acetonitrile, buffer solution (acetate or phosphate) and methanol. The injection volume was 20 μl. ROX was used as the internal standard.

The limit of detection was determined as the concentration with signal-to-noise ratio of 3.[8]

Cell Culture —— MOLT-4 cells were incubated in RPMI-1640 medium including 2.5 mg/l AMPH, 10 mg/l GM, 10 mg/l VM and 10% FBS. Human lymphocytes were incubated in RPMI-1640 medium including 2.5 mg/l AMPH, 10 mg/l GM, 10 mg/l VM and 10% human AB serum. All cells were maintained at 37°C in 5% CO_2 atmosphere.

Migration of CAM into Lymphocytes —— MOLT-4 cells (9×10^6 cells/ml) and human lymphocytes (1×10^7 cells/ml) were suspended in RPMI-1640 medium or HBSS (4 cell suspensions in total). A 2 ml aliquot of CAM HBSS solution was then added to 2 ml of each cell suspension and the cells incubated at 37°C in 5% CO_2 for 30 min. The CAM-containing lymphocytes were then separated from the CAM solution by the velocity gradient technique and the cells stored at −20°C.[16, 17] Next the CAM-containing lymphocytes were freeze-thawed, added to 200 μl of mobile phase containing 500 ng/ml of ROX and sonicated for 60 min on ice. After centrifugation ($1000 \times g$, 5 min, room temp.) and filtration (ϕ: 0.22 μm), the resultant sample solution was used for quantitative HPLC analysis, discussed below.

RESULTS AND DISCUSSION

The measurement system is shown in Fig. 1. The injected sample flowed with the mobile phase through the separation column and to the mixer. At the mixer, the separated sample solution was mixed with bipyridine complex of ruthenium ($(Ru(bpy)_3)^{3+}$) solution, which was prepared by oxidation of $(Ru(bpy)_3)^{2+}$. The reaction between $(Ru(bpy)_3)^{3+}$ and compounds with the alicyclic tertiary amine structure produced chemiluminescence, the intensity of which was measured and digitized. To determine the optimal measurement conditions, we examined the effects of the constitution of the mobile phase and the flow rate on the intensity of chemiluminescence and the separation ability of the

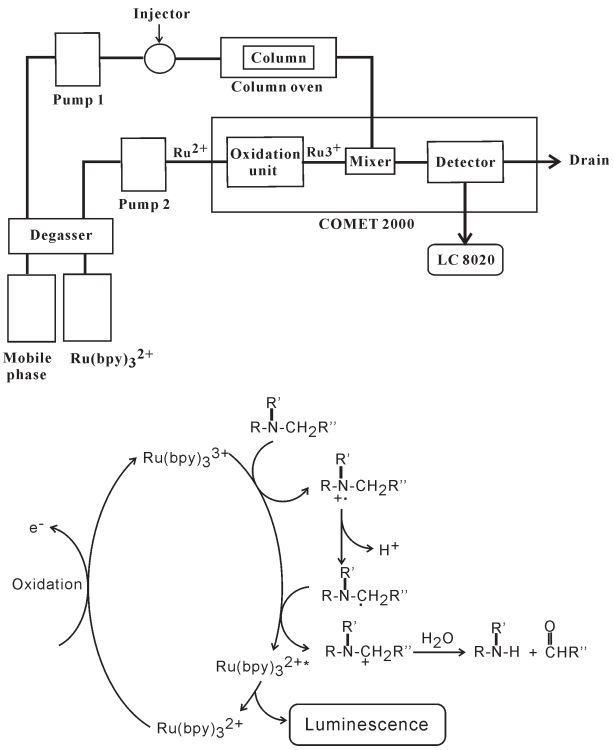

Fig. 1. Schematic Diagram of the HPLC system and chemiluminescence process

HPLC system coupled with an on-line oxidation and chemiluminescence (CL) detection system.

column.

The mobile phase was composed of acetonitrile, buffer solution and methanol. Acetate and phosphate buffer are commonly used as the buffer solution for HPLC with chemiluminescence detection using the ruthenium complex. The effect of different buffers on the intensity of chemiluminescence was investigated (Fig. 2). The ratio of constituents was fixed as follows: acetonitrile : buffer solution : methanol = 50 : 30 : 20 (v/v). We found that 100 mM phosphate buffer resulted in salt precipitation and no chemiluminescence whereas 50 mM phosphate buffer did not produce salt precipitation and chemiluminescence was detected. Furthermore, changing the buffer to acetate buffer increased the intensity of chemiluminescence, with 50 mM acetate buffer producing a higher intensity than 100 mM acetate buffer. The intensity of chemiluminescence also increased when the pH increased from 4.5 to 6.0, and decreased when the pH reached 6.5 (Fig. 2).

We also investigated whether the ratio of acetonitrile to buffer affected the retention time. Differences in retention time between CAM and ROX, and the shapes of the peaks, were observed when the ratio of acetonitrile varied from 35% to 50% (v/v).

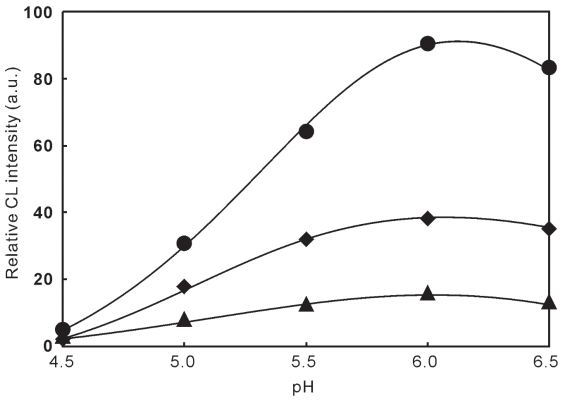

Fig. 2. Effect of the pH of the Buffer in the Mobile Phase on the CL Intensity

Concentration of CAM, 1 μg/ml; flow rate, 10 ml/min; injection volume, 20 μl. Buffers used were 50 mM phosphate buffer (closed triangle), 50 mM acetate buffer (closed circle) and 100 mM acetate buffer (closed rhombus).

The retention time of CAM decreased as the amount of acetonitrile increased. At 50% acetonitrile, the retention time was 5 min, but separation from ROX was insufficient. At 35%, 38% and 40% acetonitrile, the separation from ROX was sufficient, but width of the line indicating CAM increased. At 42% and 45% acetonitrile, separation from ROX was sufficient and a sharp line was obtained. Acetonitrile at 42% gave a higher level of separation than 45%. Based on these results, we concluded that the optimal mobile phase was acetonitrile, acetate buffer and methanol in the ratio of 42 : 38 : 20, using 50 mM acetate buffer at pH 6.0.

The flow rate of the mobile phase also affected the intensity of chemiluminescence (Fig. 3a). Of the rates tested the highest intensity was obtained at 1.0 ml/min. The flow rate of ruthenium solution also affected the intensity of chemiluminescence (Fig. 3b). The intensity increased with the decrease of the flow rate to 0.20 ml/min, but the ratio of signal/noise decrease when the flow rate was 0.20 ml/min. Therefore, the optimal flow rate of the mobile phase and the ruthenium solution was 1.0 ml/min and 0.25 ml/min, respectively. Chromatographic data are shown in Table 1.

To validate this measurement system for quantitative analysis of CAM, a calibration curve was prepared (data not shown). Linearity of the calibration curve was observed up until a CAM concentration of 20 ng on column. The regression line was taken as the straight line that passed through the origin, and the correlation coefficient was 0.999. The limit of detection was 2.0×10^{-3} ng on column. Previous studies have reported the limit of detection

of 4.2×10^{-4} ng on column[8] and the lower limit of quantitation of $6.6–15 \times 10^{-3}$ ng on column[8,9,18] for measurement of CAM in plasma by LC-MS-MS, with linearity of the calibration curve guaranteed until 4 ng on column.[8,9,18] Thus, the performance of the system used in the present study was comparable to LC-MS-MS with respect to sensitivity and measurable range.

Quantitative analysis of CAM in lymphocytes was performed using two kinds of cell and two kinds of medium. To determine the amount of CAM present in cells ROX was used as the internal standard. Figure 4 shows that the measurement system used in the present study clearly separated CAM from other compounds included in the cell suspension and from ROX. The retention time of CAM was 7.6 ± 0.4 min for every sample tested, indicating that identification process of this system is sufficient to determine the quantity of CAM in cells. The identification was not dependent on medium type or cell type. The amount of CAM that migrated into the cells was larger in RPMI-1640 than in HBSS, most likely because the addition of FBS brings the experimental conditions closer to actual biological conditions and the interaction between chlarithromycin and serum increases the hydrophobicity[19] thereby accelerating CAM uptake into cells. The higher pH of culture media also affected the results.[7]

To formulate an adequate regimen for the treatment of infectious diseases, such as *Legionella pneumonia*, the time course of the concentration of CAM in cells must be determined, as in therapeutic drug monitoring. In the present study, the concen-

Table 1. Chromatographic Data

Retention time of CAM	7.6 ± 0.4 min
Symmetry factor	1.08
Separation factor*	1.20
Theoretical plate number	4.07×10^3
Resolution	1.48

*: Roxithromycin was used as the internal standard

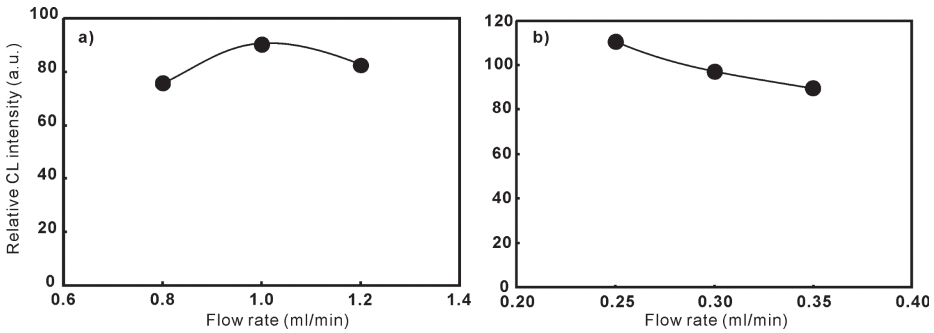

Fig. 3. Effect of Mobile Phase Flow Rate and $Ru(bpy)_3^{2+}$ Flow Rate on the CL Intensity
a) Mobile phase; b) $Ru(bpy)_3^{2+}$ solution. Concentration of CAM, 1 μg/ml; injection volume, 20 μl.

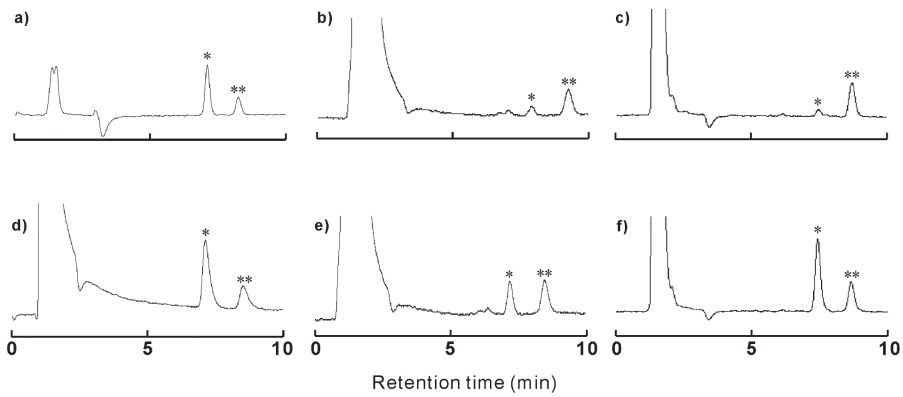

Fig. 4. Typical Chromatograms of CAM Obtained by HPLC with CL Detection
a) Standard solution; b) CAM inside lymphocytes incubated in HBSS; c) CAM inside MOLT-4 cells incubated in HBSS; d) standard solution with human serum; e) CAM inside lymphocytes incubated in RPMI-1640; f) CAM inside MOLT-4 cells incubated in RPMI-1640. *: CAM; **: ROX (internal standard).

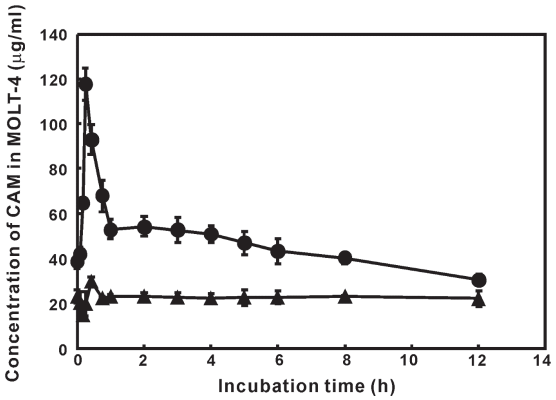

Fig. 5. Time Course of the Concentration of CAM in MOLT-4 Cells

Incubation was carried out in RPMI-1640 (closed circle) or in HBSS (closed triangle) under 5% CO_2 at 37°C. Initial conditions: concentration of CAM in cell suspension, 2 μg/ml; cell count, 1×10^7 cell/ml (MOLT-4 cells) or 9×10^6 cell/ml (lymphocytes); volume of cell suspension, 4 ml. Data express mean ± standard deviation (n = 6).

tration (μg/ml) of CAM is expressed as the amount (μg) per cell volume (ml). The amount of CAM was calculated by subtracting the residual amount from the initial amount, and cell volume was estimated using the cell diameter estimated by microscopic observation, supposing the cell to be spherical.

Figure 5 shows the time course of the concentration of CAM in MOLT-4 cells. It is suggested that macrolide antibiotics migrate into cell by active transport, but the antibiotic flow to outside the cell when the concentration of outside the cell of the antibiotics decreased.[20,21] Therefore, it is considered to find the relative maximum in the time course of the concentration of the antibiotics in cell. Our result showed the relative maximum and maximum concentration was observed at 15 min [119.56 ± 7.27 μg/ml (n = 6)] in RPMI-1640 and at 25 min [30.53 ± 1.07 μg/ml (n = 6)] in HBSS. Matsunaga reported the similar time to reach the relative maximum.[22] The maximum concentration in RPMI-1640 was much larger than that in HBSS. This tendency corresponds to the results in Fig. 4, and suggests that the performance of this measurement system is sufficiently sensitive to monitor drug concentration in cells.

We demonstrated in this study that HPLC using a post-column with tris(2,2′-bipyridine) ruthenium (III) chemiluminescence detection has comparable sensitivity to LC-MS-MS and sufficient performance to monitor the concentration of CAM in lymphocytes. Some authors reported a quantitative analysis of CAM in plasma including the report using the similar measurement system to this study.[11] It is acceptable to build a regimen to treat infectious diseases by pathogen without obligate intracellular parasites. However, for obligate intracellular parasites, results of therapeutic drug monitoring in plasma may not give useful information to treat because the change of the concentration of drug in plasma can not correspond to the concentration of drug inside cells. Then, we studied the determination the concentration of drug inside cells by the method that is feasible to introduce to medical facilities. This measurement system should also be applicable to other macrolide antibiotics and some quinolone antibacterial agents, since they all contain the alicyclic tertiary amine structure. We believe that the introduction of this HPLC system to medical facilities for analysis of intracellular antibiotic concentrations is more feasible than LC-MS-MS since both the initial and running costs of HPLC are much lower than those of LC-MS-MS and is able to develop the functional analysis suggested by some authors.[18] It is expected that this HPLC system will contribute to the formulation of effective therapeutic regimens for infectious diseases caused by obligate intracellular parasites.

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