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Determination of roxithromycin in rat lung tissue by liquid chromatography–mass spectrometry

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Abstract

A liquid chromatography–mass spectrometry (LC–MS) method for the determination of roxithromycin in rat lung tissue is described. Liquid–liquid extraction was adopted for sample preparation with recoveries from 72.5 to 76.9% at levels of 0.1, 5.0 and 20.0 μg/ml. Chromatographic separation was performed on a C_{18} column using a mixture of methanol, water and formic acid (80:20:1, v/v/v) as mobile phase delivered at a flow rate of 0.5 ml/min. Positive selected ion monitoring (SIM) mode was used for the quantification of roxithromycin at *m/z* 837.7 and clarithromycin (internal standard) at *m/z* 748.7. The linearity was obtained over the concentration range of 0.05–20.0 μg/ml and the lower limit of quantification was 0.05 μg/ml. For each QC level of roxithromycin, the intra- and inter-day precisions relative standard deviation (R.S.D.) were less than 4.1 and 7.5%, respectively, and accuracy (RE) was ±10.0%. The proposed LC–MS method has been successfully used for the determination of roxithromycin in rat lung tissue after oral administration of roxithromycin formulations to 44 SD rats. The present study demonstrates that the concentration of roxithromycin in rat lung tissues can be significantly increased by ambroxol when they are formulated in combination.

Keywords: Roxithromycin; Liquid chromatography–mass spectrometry; Rat lung tissue

1. Introduction

Roxithromycin (Fig. 1), a semi-synthetic 14-membered-ring macrolide antibiotic derived from erythromycin, is more stable than erythromycin under acidic conditions and exhibits improved clinical effects on respiratory infections [1]. It was found that ambroxol (Fig. 1), an expectorant, could increase the concentrations of such antibiotics as ampicillin, erythromycin and amoxycillin in rat lung tissues [2]. But no references are available concerning the effect of ambroxol on the concentration of roxithromycin in the lungs of rats when they are used in combination. Based on the synergic effect between erythromycin and ambroxol, it was supposed that ambroxol might have similar effect on roxithromycin. On the basis of the above supposition, a combination formulation of roxithromycin and ambroxol was developed in our laboratory.

In the preliminary phase of the development of this formulation, a biopharmaceutical test in rats was necessary in order to demonstrate if the concentration of roxithromycin in rat lung tissues can be increased by ambroxol when they are formulated in combination, which can provide the basis for the development of this combination formulation. To achieve this purpose, an analytical method of simplicity and sensitivity was required to determine the concentrations of roxithromycin in rat lung tissues. Among the analytical methods for biopharmaceutical analysis, liquid chromatography with mass spectrometry detection (LC–MS) was preferred because of its high sensitivity and selectivity. As a result of survey, several LC–MS methods related with roxithromycin were available, some of which were just used for some qual-

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itative purpose or for the determination of other drugs while using roxithromycin as internal standard, which did not provide details in terms of accuracy, precision, linearity and stability. In addition, there were several methods used for the determination of roxithromycin in biological samples [3–10] and most of them adopted tandem MS or ion-trap MS detection [3,5–7,9,10]. Our study focused on LC–single quadrupole MS because of its wider availability in ordinary laboratories as well as its sufficient sensitivity and selectivity for the present work.

The present paper describes a rapid and sensitive LC–single quadrupole MS method for the determination of roxithromycin in rat lung tissue. The developed method was validated in terms of selectivity, linearity, limit of quantification, precision and accuracy and has been successfully applied for the determination of roxithromycin in rat lung tissue after oral administration of roxithromycin formulations to 44 rats. The present study demonstrates that the concentration of roxithromycin in rat lung tissues can be significantly increased by ambroxol when they are formulated in combination.

2. Experimental

2.1. Chemicals and reagents

Roxithromycin reference standard was from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP) (Beijing, China). Clarithromycin was a gift from Shenyang Pharmaceutical University and used as internal standard (IS) in present work. A mixture suspension of ambroxol hydrochloride and roxithromycin (1:5, w/w) in PEG 400 and a suspension of roxithromycin in PEG 400 were used as test formulation and reference formulation, respectively. HPLC-grade methanol was from Tianjin Concord Reagent Company (Tianjin, China). Sodium carbonate, formic acid, *n*-hexane, dichloromethane and isopropyl alcohol were from Shenyang Chemical Reagent Company and of analytical grade.

2.2. Instrument and LC–MS conditions

HP 1100 series LC/MSD G1946D (Agilent, USA) was used.

Chromatographic separation was performed on a Diamonsil^{TM} C_{18} column (150 mm × 4.6 mm i.d., 5 μm, Dikma, China) at ambient temperature. The mobile phase consisting of a mixture of methanol, water and formic acid (80:20:1, v/v/v) was delivered at a flow rate of 0.5 ml/min. The injection volume was 20 μl.

The mass spectrometer was operated in the positive electrospray ionization (ESI) mode. The optimized ionization conditions were: nitrogen flow rate, 8.0 ml/min; gas temperature, 325 °C; nitrogen pressure, 30 psig; capillary current, 24 nA; fragmentation voltage, 150 V for roxithromycin and 170 V for clarithromycin (IS). Selected ion monitoring (SIM) mode was used for the quantification of quasi-molecular ion $[M + H]^+$ at *m/z* 837.7 for roxithromycin and *m/z* 748.7 for clarithromycin.

2.3. Animals

Sprague–Dawley rats weighing 200 g on an average were from Laboratory Animal Center of Shengyang Pharmaceutical University (certificate no. 042).

2.4. Preparation of calibration standards and quality control samples

Stock solutions (1 mg/ml) of roxithromycin and clarithromycin were separately prepared in methanol. The stock solutions were further individually diluted with methanol to give diluted standard solutions (100 μg/ml). Calibration standards of roxithromycin (0.05, 0.1, 0.2, 1.0, 5.0, 10.0 and 20.0 μg/ml) were prepared by spiking appropriate amount of the standard solutions of roxithromycin in blank rat lung homogenates. Quality control (QC) samples were prepared in blank rat lung homogenates at concentrations of 0.1, 5.0 and 20.0 μg/ml for roxithromycin. The Calibration standards and QC samples were then treated following the sample preparation procedure, as indicated in Section 2.5.

2.5. Sample preparation

Two hundred microliter aliquot of rat lung homogenate, 100 μ1 of the IS solution in methanol (13 μg/ml) and 100 μ1of mobile phase were mixed well. And then 200 μ1 of sodium carbonate aqueous solution (0.1 mol/l) was added into the mixture and shaken well. Two milliliters of a mixture of *n*-hexane–dichloromethane–isopropyl alcohol (20:10:1, v/v/v) were added and the contents were vortexed for 1 min and centrifuged for 5 min to separate the phases. The supernatant was separated and dried under a stream of nitrogen at room temperature. The residue was reconstituted with 200 μl of mobile phase and 20 μl was injected onto the LC column.

2.6. Method validation

Validation runs were conducted on three separate days. Each validation run consisted of a set of the spiked calibration standards at seven concentrations over the concentration range (each in triplicate) and QC samples at three concentrations ($n = 6$ at each concentration). The linearity of each calibration curve was determined by plotting the peak-area ratio (y) of roxithromycin to internal standard versus the nominal concentration (x) of the analyte. The calibration curves were obtained by weighted linear regression analysis ($1/x^2$ weighing factor). Calibration standards and QC samples were

evenly distributed throughout the run. The results from QC samples in three runs were used to evaluate the accuracy and precision of the method.

The selectivity of the method was investigated by comparing chromatograms of blank rat lung tissue, calibration standards spiked with roxithromycin (5 μg/ml) and the IS (13 μg/ml) and rat lung tissue samples after oral administration of a suspension solution of roxithromycin in PEG 400.

The extraction recoveries of roxithromycin were determined at low, medium and high concentrations by comparing the responses from QC samples spiked before extraction with standard solutions without extraction. The recovery is calculated by the formula: recovery (%) = (detector response of extracted analyte/detector response for non-extracted analyte) × 100, where detector response is the area of the chromatographic peak for extracted or non-extracted analyte divided by the area of the chromatographic peak for the internal standard added.

Sample stability was determined by analyzing QC samples containing roxithromycin of 0.1, 5.0 and 20.0 μg/ml after sample preparation and exposed to ambient temperature over a time period of 4 h.

2.7. Application of the LC–MS method

The LC–MS method has been successfully applied for the determination of roxithromycin in rat lung tissues after oral administration of test or reference formulations to rats. Forty-four SD rats were divided randomly into two groups, test group and reference group, with 22 rats in each group. Test and reference formulations were orally administered at a dose 10 mg/kg (on the basis of roxithromycin) to rats in test group and reference group after 12 h fast, respectively. Animals were allowed free access to food and water. Six hours after administration, rats were sacrificed and lung tissues were excised and weighed. A portion of accurately weighed tissues (1 g) was homogenized in 2 ml methanol and centrifuged for 10 min. The supernatant was separated and stored at −20 °C until analysis. The research complied with national legislation on the care of use of animals and with related codes of practice.

3. Results and discussion

3.1. Method development

Sample preparation is usually required for the determination of pharmaceuticals in biological samples owing to complex matrices such as fluids, tissues and organs in order to remove possibly interfering matrix components and increase the selectivity and sensitivity. Liquid–liquid extraction (LLE) was a widely adopted method and often achieved satisfactory extraction recoveries of analytes from biological samples. The LLE procedures reported differed greatly in extraction solvents (diethyl ether, dichloromethane) and samples (urine, flounder muscle, broiler and rat tissues). In present work, a mixture of *n*-hexane–dichloromethane–isopropyl alcohol (20:10:1, v/v/v) was finally used for the extraction of roxithromycin from rat lung tissue, which produced a clean chromatogram for a blank tissue sample and offered satisfactory extraction recoveries for the analyte from 72.5 to 76.9% at levels from 0.1 to 20.0 μg/ml.

A Diamonsil^{TM} C_{18} column (150 mm × 4.6 mm i.d., 5 μm) was used for the chromatographic separation. For mobile phase, a mixture of methanol, water and formic acid (80:20:1, v/v/v) was found to be optimal for this work, which provided symmetric peak shapes of the analyte and internal standard as well as short run time. For the selection of internal standard, structural analogs became the first choice. Several macrolides were tried and clarithromycin was finally used as the internal standard in this work.

ESI in positive mode was used in this work. Parameters involving capillary temperature, vaporizer temperature and flow rate were optimized to obtain the protonated molecules of the analytes. The fragmentation voltage was optimized at 150 eV for roxithromycin and 170 eV for clarithromycin. Selected ion monitoring was used for the quantification of roxithromycin at *m/z* 837.7 and clarithromycin at 748.7. Two detection channels were adopted, channel 1

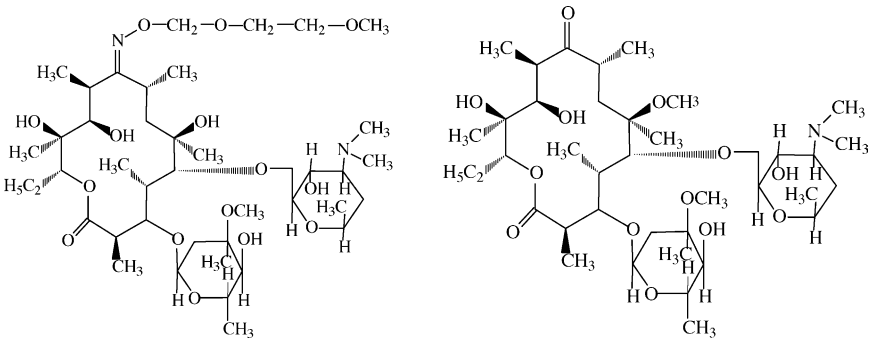

Fig. 1. Chemical structures of roxithromycin (left) and clarithromycin (right).

(MSD1) for roxithromycin and channel 2 (MSD2) for clarithromycin.

3.2. Selectivity

The results for selectivity are shown in Fig. 2. The quasi-molecular ions for the quantification were *m/z* 837.7 for roxithromycin and *m/z* 748.7 for clarithromycin. Fig. 2 indicates no significant interferences from endogenous substances in rat lung with the analyte and internal standard.

3.3. Linearity and limit of quantification

Weighted linear regression analysis was used to construct the calibration curve. Representative regression equation for the calibration curve was $y = 0.0505x - 0.0033$ ($r = 0.9955$, $n = 7$) over the concentration range of 0.05–20.0 μg/ml for roxithromycin. The lower limit of quantification (LLOQ) for roxithromycin was found to be 0.05 μg/ml. At this level, the intra- and inter-day precisions relative standard deviation (R.S.D.) were 6.3 and 15.5%, respectively, and accuracy

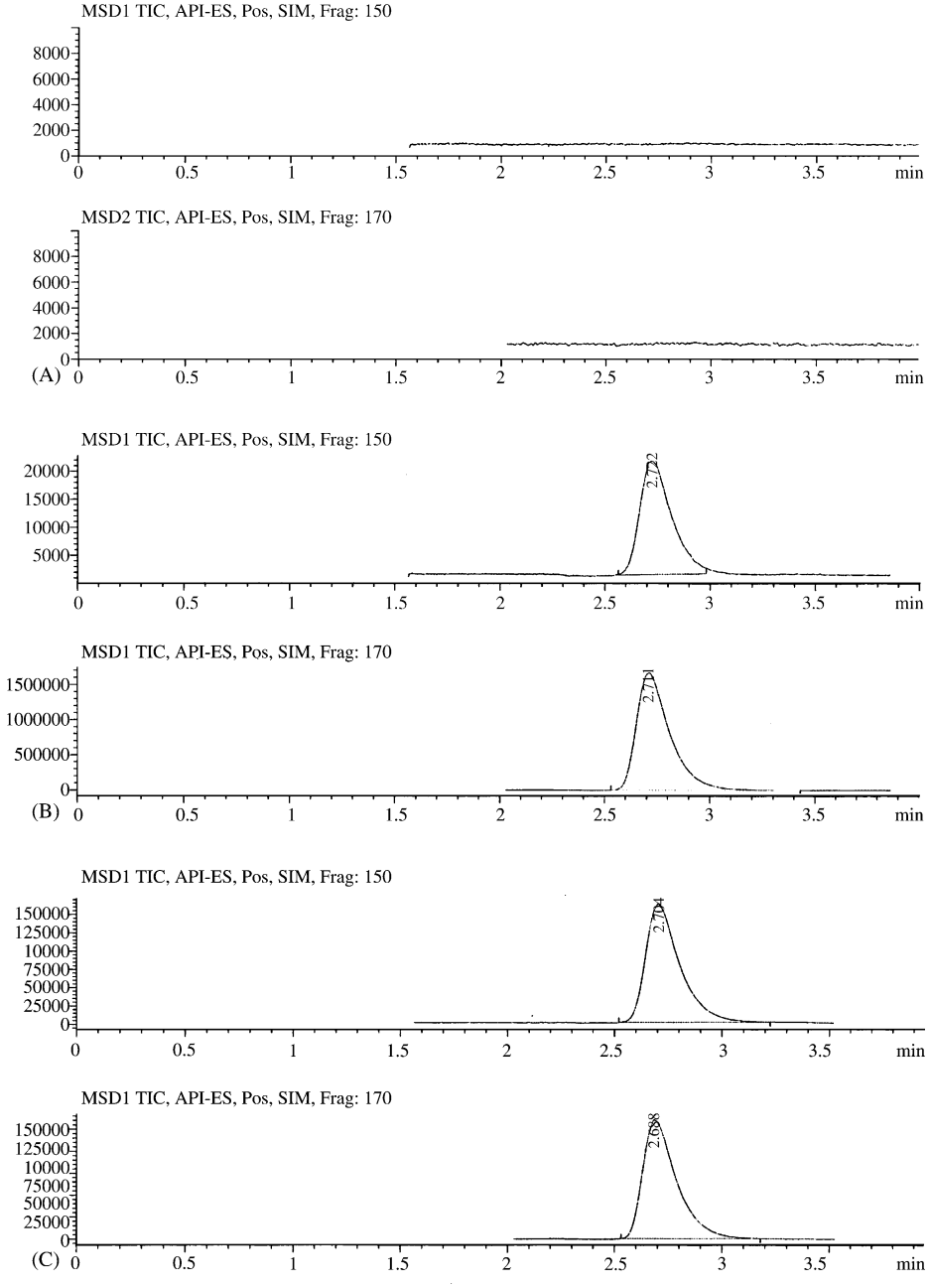

Fig. 2. Representative SIM chromatograms of: (A) blank rat lung homogenate sample; (B) blank rat lung homogenate sample spiked with roxithromycin (5 μg/ml) and clarithromycin (IS) (13 μg/ml); (C) a rat lung sample after oral administration of a suspension solution of roxithromycin and ambroxol hydrochloride (5:1, w/w) in PEG 400. Two channels were used for the quantification, MSD1 for roxithromycin (t_R = 2.7 min) and MSD2 for clarithromycin (IS) (t_R = 2.7 min).

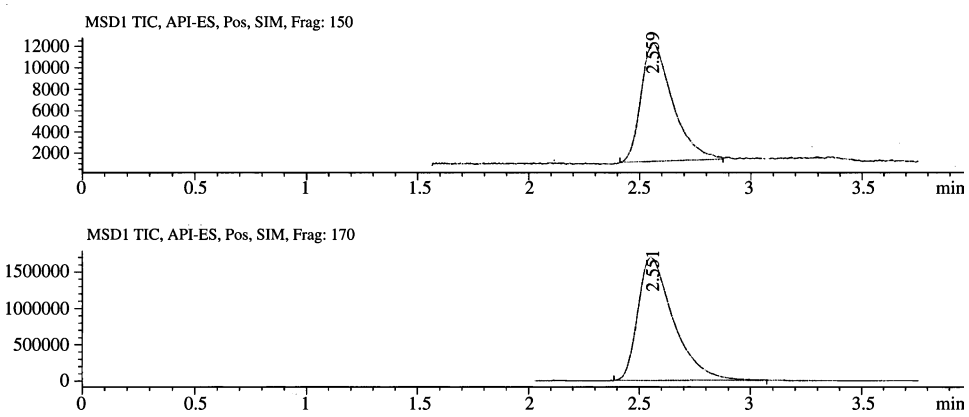

Fig. 3. Representative SIM chromatograms of blank rat lung homogenate sample spiked with roxithromycin (0.05 μg/ml) and clarithromycin (IS) (13 μg/ml). Two channels were used for the quantitation, MSD1 for roxithromycin (t_R = 2.7 min) and MSD2 for clarithromycin (IS) (t_R = 2.7 min).

Table 1
Accuracy and precision for the determination of roxithromycin in rat lung tissues (three runs, six replicates per run)

Nominal concentration *C* (μg/ml)	Observed concentration *C* (μg/ml)	Intra-day R.S.D.[a] (%)	Inter-day R.S.D. (%)	Relative error (%)
0.10	0.10	4.1	7.5	4.7
5.00	4.84	2.6	4.6	−3.1
20.00	21.99	1.7	1.9	10.0

Relative error: RE (%) = [(mean concentration − nominal concentration)/nominal concentration] × 100.
[a] Relative standard deviation.

(RE) was 2.2%. Representative SIM chromatogram with roxithromycin at LLOQ level is shown in Fig. 3.

3.4. Accuracy and precision

The accuracy and precision of the method were evaluated based on the data from QC samples at three concentrations (0.1, 5.0 and 20.0 μg/ml) for roxithromycin in three validation runs (Table 1). The intra- and inter-day precision was expressed as the relative standard deviation (R.S.D.). The accuracy was determined by calculating the percentage deviation observed in the analysis of QC samples and expressed in the relative error (RE). Table 1 shows that for each QC level of roxithromycin, the intra- and inter-day precisions (R.S.D.) were less than 4.1 and 7.5%, respectively, and accuracy (RE) was ±10.0%, indicating the acceptable accuracy and precision of the present LC–MS method for the determination of roxithromycin in rat lung tissue.

3.5. Extraction recovery

The extraction recoveries of roxithromycin from rat lung tissue were 74.4 ± 1.9, 72.5 ± 1.9 and 76.9 ± 1.3% at concentration levels of 0.1, 5.0 and 20.0 μg/ml, respectively.

3.6. Stability

The stability of roxithromycin in rat lung homogenate at room temperature after sample preparation was determined. Roxithromycin was found to be stable for at least 4 h at ambient temperature with an accuracy (RE) within ±10.0% and precision (R.S.D.) less than 5.3% at three levels of QC samples.

3.7. Application of the developed LC–MS method

The LC–MS method has been successfully used for the determination of roxithromycin in rat lung tissue at 6 h after oral administration of a test or reference formulation to 44 rats. The mean concentrations (mean ± S.D.) for the analyte from the test group and reference group were 4.06 ± 1.71 and 3.20 ± 0.95 μg/ml, which proved to be significantly different by two-sided *t*-test ($p < 0.05$). The concentration in test group is 26.9% higher than that in reference group, indicating that ambroxol can significantly increase the concentration of roxithromycin in rat lung tissues when they are formulated in combination. The results justified the combination formulation of roxithromycin and ambroxol hydrochloride and can provide the basis for the further study of the formulation in healthy volunteers.

4. Conclusions

A simple and sensitive liquid chromatography–single quadrupole mass spectrometry was developed for the determination of roxithromycin in rat lung tissues. The present LC–MS method adopted a simple liquid–liquid extraction procedure for the sample preparation and offered sufficient sensitivity and satisfactory selectivity and can be used for

the pharmacokinetic studies of roxithromycin formulation products in animals and volunteers. Besides, the results of the present study demonstrate that the concentration of roxithromycin in rat lung tissues can be significantly increased by ambroxol when they are formulated in combination, which provides the basis for clinical trials.

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