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Determination of roxithromycin in human plasma by high-performance liquid chromatography with spectrophotometric detection

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Abstract

A simple and reproducible method for the determination of roxithromycin in human plasma is presented. This method is based on liquid–liquid extraction with hexane–isoamylalcohol (98:2, v:v) and reversed-phase chromatography with spectrophotometric detection at 220 nm. The mobile phase consists of methanol–15 m*M* dihydrogen potassium phosphate (70:30, v:v), pH of the aqueous part of the mobile phase is 6.0. The column is operated at 60°C. Clarithromycin is used as the internal standard. The limit of quantitation is 0.5 μg/ml and the calibration curve is linear up to 30 μg/ml. Within-day and between-day precision expressed by relative standard deviation is less than 5% and inaccuracy does not exceed 9%. The assay was used for pharmacokinetic studies.

Keywords: Roxithromycin

1. Introduction

Roxithromycin is a semisynthetic macrolide antibiotic derived from erythromycin. It is well and rapidly absorbed with long elimination half-time and gives plasma levels that are higher than those of erythromycin [1].

Several chromatographic methods have been reported for determination of roxithromycin in biological fluids. The sample pre-treatment consisted of liquid–liquid extraction [2,3] or was based on column-switching [4], chromatography on reversed phase columns was used and electrochemical detection was employed in all these methods.

In comparison with other macrolides roxithromycin has somewhat higher absorbance at lower wavelengths. Therefore a method with spectrophotometric detection should be feasible, because a limit of quantitation 0.5 μg/ml is sufficient for pharmacokinetic studies [5].

The aim of the present study was to develop a sufficiently sensitive method for roxithromycin determination with spectrophotometric detection.

2. Experimental

2.2. Chemicals

Methanol (LiChrosolv, for chromatography), hexane (for spectroscopy, Uvasol) and potassium di-

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hydrogenphosphate (analytical grade) were manufactured by Merck (Darmstadt, Germany). Isoamylalcohol was Fluka Chemie AG product (Buchs, Switzerland). Roxithromycin (purity 99.2%) was obtained from Léčiva (Prague, Czech Republic). Clarithromycin (internal standard) was obtained in a local pharmacy in the form of the coated tablets (250 mg tablets, Klacid, Abbott SpA, Campoverde di Aprilia, Italy).

2.2. Apparatus

All HPLC instruments were obtained from Thermo Separation Products (Riviera Beach, FL, USA). The system consisted of the membrane degasser, pump ConstaMetric 4100, automatic sample injector AS 3000, spectrophotometric detector UV2000 and datastation with PC1000 software, version 2.5. The separation was performed on Nucleosil 100-3 C18 particle size 3 μm, 150×4.6 mm I.D. column (Watrex, Prague, Czech Republic). A pre-column 10×4 mm I.D. packed with Nucleosil 120-5 C18 particle size 5 μm was used.

The mobile phase consisted of methanol–15 m*M* potassium dihydrogenphosphate buffer (70:30, v:v), pH of the buffer was adjusted to 6.0 with potassium hydroxide. The flow-rate of the mobile phase was 1.2 ml/min at 60°C. The spectrophotometric detector was operated at 220 nm with the time constant 1 s.

2.3. Standards

Stock solutions of roxithromycin were made by dissolving approximately 30 mg in 10 ml of methanol. Separate solutions were prepared for calibration curve and quality control samples. Further solutions were obtained by serial dilutions of stock solutions with methanol. These solutions were added to drug-free plasma in volumes not exceeding 1% of the plasma volume.

One Klacid tablet was disintegrated using pestle and mortar and dissolved in 60 ml of methanol in the ultrasonic bath. The mixture was centrifuged at 2600 *g* for 10 min and the supernatant was used as the internal standard. The solution was stable for one month, all samples and standards were analyzed with the same solution of internal standard.

All solutions were stored at −18°C and protected from light.

2.4. Preparation of the sample

The samples were stored in the freezer at −18°C and allowed to thaw at room temperature before processing.

Ten μl of internal standard solution (41.7 μg/ml of clarithromycin) were added to 1 ml of plasma, the tube was briefly shaken. One hundred μl of 1 *M* NaOH was added and the tube was shaken again. Then the mixture was vortex mixed with 4 ml of hexane:isoamylalcohol (98:2, v:v) 60 s at 2000 rpm. The tube was centrifuged 5 min at 2600 *g*, the upper organic phase was transferred to another tube and evaporated to dryness under the stream of nitrogen at 45°C. The residue was dissolved in 200 μl of the mixture methanol–15 m*M* potassium dihydrogenphosphate buffer (1:1, v:v). The sample was transferred to the polypropylene autosampler vial and 40 μl were injected into the chromatographic system.

2.5. Calibration curves

The calibration curve was constructed in the range 0.5–29.6 μg/ml to encompass the expected concentrations in measured samples. The concentrations of individual standards were 0.506, 0.913, 3.00, 8.94, 27.1 and 29.6 μg/ml. The calibration curves were obtained by weighted linear regression (weighting factor $1/y^2$ was selected, because the standard deviation of measured concentration depends on roxithromycin concentration): the ratio of roxithromycin peak height to clarithromycin peak height was plotted vs. ratio of roxithromycin concentration to that of internal standard in ng/ml. The suitability of the calibration model was confirmed by back-calculating the concentrations of the calibration standards.

2.6. Limit of quantitation

Limit of quantitation (LOQ) was defined as the lowest concentration at which the precision expressed by relative standard deviation is better than 20% and accuracy expressed by relative difference of

the measured and true value is also lower than 20%. Six identical samples were analyzed for the determination of LOQ.

3. Results and discussion

3.1. Chromatography

Roxithromycin exhibits weak absorbance at higher wavelengths (>235 nm), thus mobile phase containing acetonitrile would be the first choice when spectrophotometric detection at low wavelengths is employed. Nevertheless methanol was used as the organic solvent because it significantly reduced peak tailing. Higher analysis temperature (60°C) improved column efficiency for macrolides and enabled faster analysis due to lower mobile phase viscosity and higher flow-rate.

Under the described chromatographic conditions the retention time of roxithromycin and clarithromycin was 8.5 and 10.2 min, respectively. The column efficiency expressed by the number of theoretical plates was 2500, peak asymmetry measured at 5% of the peak height was better than 1.5 for both compounds and the resolution of both compounds was 2.0.

The method selectivity was demonstrated on six blank plasma samples obtained from healthy volunteers: the chromatograms were found to be free of interfering peaks. The typical chromatogram of blank plasma is shown in Fig. 1. The chromatogram of a plasma sample 4 h after administration of 300 mg of roxithromycin to a volunteer is shown in Fig. 2. The concentration of roxithromycin was 6.74 μg/ml.

3.2. Sample preparation

Poor recoveries were achieved with dichloromethane which was used in previous assays as the extraction solvent. Liquid–liquid extraction with hexane:isoamylalcohol 98:2 exhibited nearly total recovery (90±3%), nevertheless attempts to develop the method without an internal standard failed due to unsatisfactory precision of the method. Structurally similar macrolide antibiotics are suitable candidates for internal standard, especially erythromycin and clarithromycin due to the similar chromatographic behaviour. Finally, clarithromycin was chosen because commercially available erythromycin contained interfering impurities.

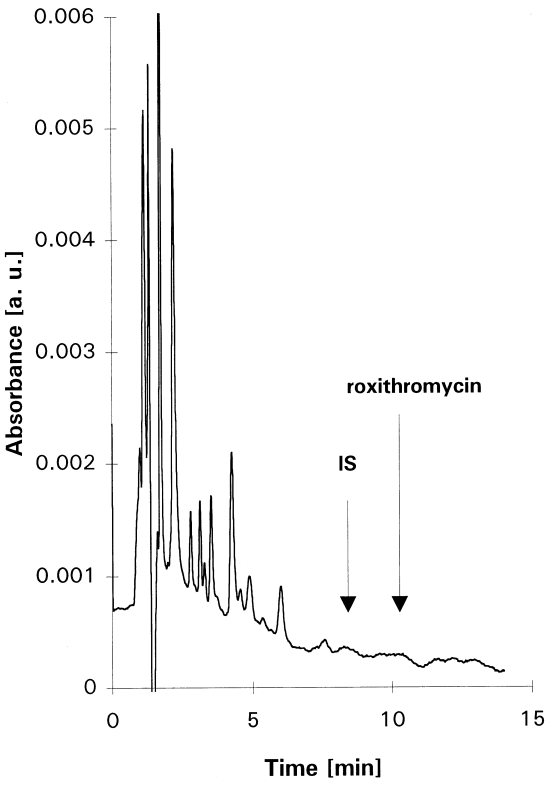

Fig. 1. Typical chromatogram of drug-free human plasma. The arrows indicate the retention time of roxithromycin and clarithromycin (IS).

3.3. Linearity and limit of quantitation

The calibration curves were linear in the studied range. The calibration curve equation is $y=bx+c$, where y represents the roxithromycin peak height to clarithromycin peak height ratio and x represents the ratio of roxithromycin concentration to that of internal standard. The mean equation (curve coefficients±standard deviation) of the calibration curve ($N=10$) obtained from 6 points was $y=2.677(\pm0.077)x+0.0032(\pm0.0072)$ (correlation coefficient $r=0.9990$). The mean difference between true and back-calculated concentration of the calibration standard was 3.2% (range 1.7 to 5.9%); this indicates the suitability of the calibration model.

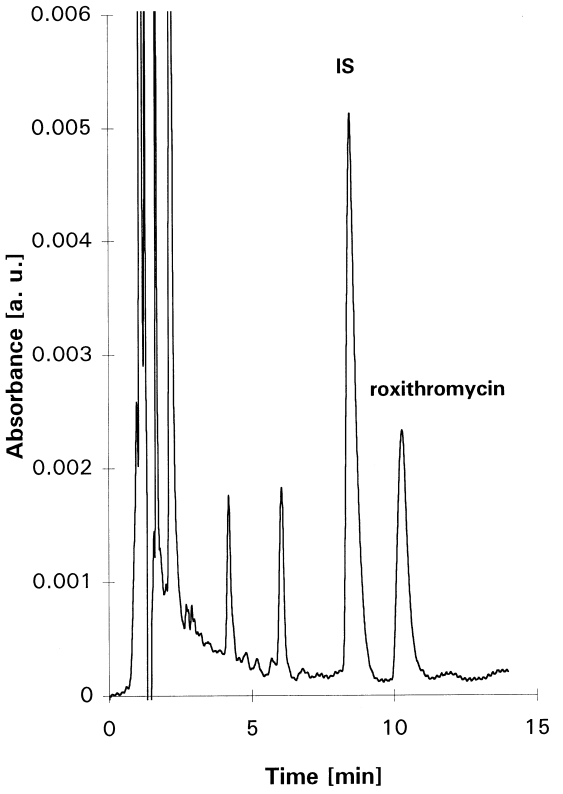

Fig. 2. Chromatogram of a plasma sample from a volunteer 4 h after administration of 300 mg of roxithromycin. The respective concentration was 6.74 μg/ml.

The limit of quantitation was 0.5 μg/ml. The precision, characterised by the relative standard deviation, was 16.1% and accuracy, defined as the deviation between the true and the measured value expressed in percents, was −14.6% at this concentration ($N=6$).

3.3.1. Intra-day precision

Intra-day precision of the method is illustrated in Table 1. Six sets of quality control samples (low, medium and high concentration) were analysed with calibration samples on one day. Both precision and accuracy were better than 9% at all levels.

3.3.2. Inter-day precision and accuracy

Inter-day precision and accuracy were evaluated by processing a set of calibration and quality control samples (3 levels analysed twice, results averaged for statistical evaluation) on six separate days. The samples were prepared in advance and stored at −18°C. The respective data are given in Table 2. The precision was better than 8% and the inaccuracy did not exceed 9% at all levels.

Table 1
Intra-day precision and accuracy

N[a]	Concentration (μg/ml)			
	Added	Measured	Bias	RSD
6	0.945	0.862	−8.8%	8.9%
6	3.85	3.82	−0.6%	2.9%
6	24.97	24.57	−1.6%	1.5%

[a] N=number of samples.

3.3.3. Stability study

3.3.3.1. *Freeze and thaw stability*. Ten millilitres of a low and high concentration sample were prepared. The solutions were stored at −18°C and subjected for 3 thaw and freeze cycles. During each cycle triplicate 1 ml aliquots were processed, analysed and the results averaged. The results are shown in Table 3. The concentrations found are within the allowed limit ±15% of nominal concentration. Also changes relatively to the first cycle are less than 6%, indicating no significant substance loss during repeated thawing and freezing.

3.3.3.2. *Processed sample stability*. Two sets of samples (0.945 μg/ml as a low and 24.97 μg/ml as a high concentration of roxithromycin) were analysed on one day and left in the autosampler at ambient temperature. The samples were analysed using a freshly prepared calibration sample five days later. The results are presented in Table 3. The

Table 2
Inter-day precision and accuracy

N[a]	Concentration (μg/ml)			
	Added	Measured	Bias	RSD
6	0.945	0.866	−8.4%	7.7%
6	3.85	3.87	0.5%	4.8%
6	24.97	24.74	−0.9%	4.1%

[a] N=number of days.

Table 3
Stability of the samples

Freeze and thaw stability							
Sample C [μg/ml]	N^a	Cycle 1		Cycle 2		Cycle 3	
		Measured	Bias	Measured	Bias	Measured	Bias
2.06	3	1.80	−13%	1.89	−8.1%	1.85	−10%
24.97	3	24.14	−3.3%	25.98	4.0%	25.16	0.8%

Processed sample stability				
Sample	N^a	Conc. found [ng/ml]	RSD	Difference
New	6	0.872	14.2%	
5 days old	6	0.859	4.3%	−1.5%
New	6	24.57	1.5%	
5 days old	6	24.63	0.9%	0.2%

Long-term stability				
C (ng/ml)	N^a	Conc. found [ng/ml]	RSD	Bias
1.99	6	1.97	7.8%	−1.1%
25.13	6	25.34	6.8%	0.8%

[a] N=number of samples.

processed samples are stable at room temperature for 5 days.

3.3.3.3. *Long term stability*. Two sets of samples (low and high concentration of roxithromycin) were stored in the freezer at −18°C for six weeks. The samples were then analysed using freshly prepared calibration samples. The results are presented in Table 3. The samples are stable at −18°C for six weeks.

3.4. Application to biological samples

The proposed method was applied to the determination of roxithromycin in plasma samples for the purpose of the bioequivalence study. Plasma samples were periodically collected up to 60 h after oral administration of one tablet containing 300 mg of roxithromycin to 26 healthy male volunteers. Fig. 3 shows the mean plasma concentrations of roxithromycin. The plasma level of roxithromycin reached a maximum 1.5 h after the administration and thereafter the plasma level declined with an elimination half-time of ca. 12 h. These values agree with previously published reports [1]. The extrapolated fraction of the AUC from 0 to infinity ac-

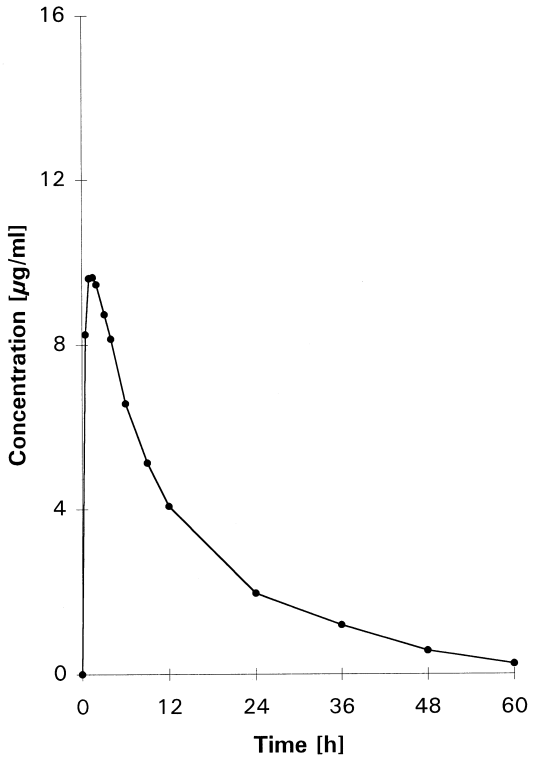

Fig. 3. Mean plasma concentrations of roxithromycin after 300 mg single oral dose (26 healthy volunteers).

counted only for 8% which indicates a suitability of the analytical method for pharmacokinetic studies.

4. Conclusions

The described method allows determination of roxithromycin in human plasma with basic instrumentation of the chromatographic laboratory. The routine operation of a spectrophotometric detector is simpler because no detector cell cleaning is required as in the electrochemical detection and the detector response is more stable. The precision and accuracy of the method are fully comparable with the previously published procedures and the sensitivity of the assay is sufficient to follow the pharmacokinetics of this drug.

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