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Short communication

Determination of roxithromycin in human plasma by HPLC with fluorescence and UV absorbance detection: Application to a pharmacokinetic study

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Abstract

A selective HPLC method with fluorescence detection for the determination of roxithromycin (ROX) in human plasma was described. After solid-phase extraction (SPE), ROX and erythromycin (internal standard, I.S.) were derivatized by treatment with 9-fluorenylmethyl chloroformate (FMOC-Cl). Optimal resolution of fluorescence derivatives of ROX and I.S. was obtained during one analytical run using reversed phase, C_{18} column. The mobile phase was composed of potassium dihydrogenphosphate solution, pH 7.5 and acetonitrile. Fluorescence of the compounds was measured at the maximum excitation, 255 nm and emission, 313 nm, of ROX derivatives. Validation parameters of the method were also established. After SPE, differences in recoveries of ROX and erythromycin from human plasma were observed. The linear range of the standard curve of ROX in plasma was 0.5–10.0 mg/l. The validated method was successfully applied for pharmacokinetic studies of ROX after administration of a single tablet of ROX.

Keywords: Macrolide antibiotics; FMOC derivatives; Solid-phase extraction; UV and fluorescence detection; Pharmacokinetics

1. Introduction

Roxithromycin (ROX)—9-{*O*-[(2-methoxyethoxy)-methyl]-oxime}-erythromycin is a semi-synthetic, 14-membered ring macrolide antibiotic, in which the erythronolide A lactone ring has been altered to prevent inactivation in the gastric environment. It has proven clinical efficacy against some *Staphylococcus* spp. and many *Streptococcus* spp. [1]. Weak UV absorption of macrolides in the low wavelength range (<220 nm) means that the RP-HPLC method requires the use of electrochemical detection for determination of macrolide antibiotics in biological matrices [2–5]. However, the published methods were not always completely validated for use in pharmacokinetic studies [2–4]. Nevertheless, the HPLC method with spectrophotometric detection has been proposed [6]. Very sensitive HPLC methods, based on mass spectrometry detection, were reported for determination of ROX in some tissues [7–9]. A HPLC method with sensitive fluorescence detection was also suggested [10]. However, the proposed linear range of the calibration curve beginning at 3.24 mg/l was insufficient for bioavailability studies of ROX after administration at low doses. Moreover, no data are available which would confirm the usefulness of the method in in vivo conditions.

The present paper describes a rapid, sensitive and precise HPLC method with fluorescence detection for quantification of ROX after derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl). Erythromycin was used in the study as the internal standard (I.S.). UV and fluorescence intensity of ROX and erythromycin derivatives were estimated and analytical differences between the two analyzed macrolide antibiotics were discussed. Moreover, optimal conditions have been established for solid-phase extraction (SPE) with cartridges filled with C_{18} stationary phase for isolation of ROX and I.S. from plasma. The assay, validated in terms of selectivity, linearity, limit of quantification, yield for SPE, stability, precision and accuracy, was successfully applied in human pharmacokinetic studies after administration of a single tablet containing 150 mg of ROX.

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2. Experimental

2.1. Materials

ROX as a reference substance (Alembic Limited, India) and erythromycin as a reference substance (PPH Galfarm Ltd., Kraków, Poland) were used. FMOC-Cl and methanol of HPLC grade were purchased from Merck (Darmstadt, Germany). Acetonitrile (J.T. Baker Mallinckrodt, Deventer, Holland) was of HPLC grade. Sodium hydroxide (P.O.Ch., Gliwice, Poland) was also used. Disodium hydrogen phosphate anhydrous was obtained from Fluka (Buchs, Switzerland) and potassium dihydrogenphosphate was purchased from Xenon (Łódź, Poland). Demineralised water (0.1 μS/cm) was always used (Seradest USF 1900, USF Seral, Germany).

2.2. Chromatographic conditions

The mobile phase was prepared by mixing 700 ml of acetonitrile with 300 ml of a 0.05 mol/l solution of potassium dihydrogenphosphate in water, adjusted to pH 7.5 with a 10% sodium hydroxide solution. Determination of ROX and I.S. in human plasma was performed in a chromatograph model HP 1100 (Hewlett-Packard, Waldbronn, Germany). The HPLC system consisted of a quaternary pump model G1311A set at a flow rate of 2 ml/min, a column oven model G1316A set at 40 °C and a fluorescence detector model HP 1046A and G1321A-1100, set at an excitation wavelength (Ex) of 255 nm and emission wavelength (Em) of 315 nm. The samples (100 μl) were injected using autosampler model G1313A. The separation was performed on a 125 × 4.6 mm i.d. LiChrospher RP-18e column packed with 5 μm particles, with a guard column (LiChrospher RP-18e), both from Merck.

2.3. Sample preparation

Stock solutions of ROX and I.S. were prepared with 1 g/l, each in methanol. Then, standard solutions: 5.0, 7.5, 15.0, 20.0, 50.0, 75.0 and 100.0 mg/l of ROX and 200.0 mg/l of I.S. were prepared also in methanol. The volume of 50 μl aliquots of the sample was transferred to a glass vial containing 0.5 ml blank human plasma. The resulting plasma samples containing: 0.5, 0.75, 1.5, 2.0, 5.0, 7.5 and 10.0 mg/l of ROX and 20.0 mg/l of I.S. were processed according to the SPE procedure described below.

2.4. Extraction procedure

Yields for SPE of ROX were determined at four different values of pH. Therefore, four series of plasma samples with ROX and I.S. were prepared. The volume of 0.5 ml of a phosphate buffer, pH 7.5 (prepared as a mixture of 85.2 ml of 1/15 M Na_2HPO_4 and 14.8 ml 1/15 M KH_2PO_4) was added to the first series. The subsequent series of plasma samples with pH 9.0, 10.0 and 12.5 were obtained by adding an adequate volume of 1 M solution of sodium hydroxide. The samples were transferred into C_{18} SPE Bakerbond cartridges (J.T. Baker Mallinckrodt, Deventer, Holland). The absorbed analytes were washed with water and eluted with methanol. The organic liquid was evaporated to dryness at 40 °C.

2.5. Derivatization conditions

The residue was dissolved in 200 μl aliquots of acetonitrile and the sample was transferred to a glass reaction vial. A 100 μl aliquots of a 2.5 mg/l FMOC-Cl in acetonitrile and 100 μl aliquots of a phosphate buffer, pH 7.5 were added. The sample was incubated at 40 °C for 40 min. After derivatization, 100 μl aliquots of the solution was injected onto the chromatographic system.

2.6. Validation parameters

For the examination of specificity of the method, drug-free human plasma sample was tested. The sample was prepared according to the extraction and derivatization procedures described in Sections 2.4 and 2.5. Then, a chromatogram of a sample containing ROX and I.S. was compared with a chromatogram of the blank plasma.

Linearity of the calibration curve was estimated for the peak area of ROX to I.S. ratio as a function of the analyte concentration in plasma ranging from 0.5 to 10.0 mg/l.

The limit of detection (LOD) for ROX was determined as a signal-to-noise (S/N) baseline ratio of 11:1. The lower limit of quantification (LLOQ) was defined as the lowest concentration of ROX of the calibration curve at which the coefficient of variation (CV) was $\leq$15% of the nominal concentration.

Intra-day precision of the elaborated method was determined for 1.5, 5.0 and 10.0 mg/l ROX concentrations in human plasma, for six samples of each concentration. Inter-day precision was estimated for all concentrations within the calibration curve range. The precision was expressed as CV. Accuracy was estimated for the same range of ROX concentrations as for evaluation of precision of the method. The yield for SPE of 1.5 and 10.0 mg/l ROX concentrations was evaluated. The first series consisted of six or twelve 0.5 ml blank plasma samples, each spiked with 50 μl aliquots of solution containing ROX at concentration of 15.0 or 100.0 mg/l ROX and I.S of 200 mg/l. The samples were extracted according to the procedure described in Section 2.4. Then, six or twelve blank plasma samples of second series were supplemented with I.S. only and ROX was added after the above SPE procedure. The yields for SPE were calculated as the peak area ratio of ROX to I.S., from the spiked and non-extracted samples.

The stability of six samples, three of high (7.5 mg/l) and three of low (1.5 mg/l) concentration was analyzed before and after three freeze and thaw cycles and during storage in autosampler at ambient temperature. The samples were prepared according to the procedure described above.

2.7. In vivo application

The utility of the worked out methodology was demonstrated in pharmacokinetic studies on ROX in 26 healthy volunteers,

after administration of a tablet containing 150 mg ROX (Rulid, Roussel Laboratories, UK). Thirteen male and 13 female healthy volunteers (27 ± 6 years old, 69 ± 12 kg body weight) were selected for the study. The investigation was approved by the Bioethical Committee attached to the University of Medical Sciences in Poznań and performed in compliance with Good Clinical Practise (GCP) based on the Declaration of Helsinki. The plasma ROX concentrations were used to calculate pharmacokinetic parameters by means of Topfit 2.0 software package (Gustav Fischer, Stuttgard, 1993). The calculation was based on the open one compartment model. The elimination rate constant (k_{el}) was estimated by linear segment of the log plasma drug concentration versus time data. The elimination half-life ($t_{0.5}$) was calculated from $0.693/k_{el}$. Total area under concentration–time curve ($AUC_{0\rightarrow\infty}$) was obtained by trapezoidal rule with extrapolation to infinity by the use of C_{last}/k_{el}.

3. Results and discussion

3.1. Derivatization procedure and HPLC

ROX and other macrolides have weak UV absorbance and therefore generally HPLC methods with more sensitive electrochemical (EC) [2–5] or recently mass spectrometry (MS) [8,9] or tandem MS [7] detectors with electrospray ionization mode have been applied. HPLC with sensitive fluorescence (FL) detection can provide an alternative to both these methods. However, ROX and erythromycin have no fluorescence properties. Therefore, the macrolides have to undergo derivatization with the use of fluorogenic agents to enable determination of low concentrations of the compounds in biological fluids. Sastre et al. [10] reported the reaction between FMOC-Cl and hydroxyl groups of macrolides, and this reaction was also chosen in the present investigations. Chromatogram analysis of the UV absorbance spectrum and FL emission spectrum of ROX and erythromycin derivatives injected into the HPLC column demonstrated considerable differences in intensity between the two analytes. The peak areas of the erythromycin derivative, with a retention time of 5.1 min, obtained with UV and FL detection were very similar, but in the case of the ROX derivative, with a retention time of 10.7 min, notable differences were observed. Peak areas obtained using the FL detector, were three times greater than those resulting from UV detection (Fig. 1). It could be concluded that for both ROX and erythromycin, derivatization with FMOC-Cl significantly improved quantification of the analytes using UV detection compared to quantification of pure, underivatized analytes. Moreover, application of fluorescence detection for determination of the ROX derivative considerably improved its limit of quantification to below 1 mg/l in comparison with erythromycin FMOC, where UV detection could be used interchangeably with fluorescence detection.

3.2. Optimization of extraction

Liquid–liquid extraction with *tert*-butyl methyl ether [3,4], mixtures of hexane with other solvents [6,9], dichloromethane [8] or diethyl ether [10] was mainly applied for extraction of ROX and other macrolides. SPE cartridges have occasionally been used for isolation of macrolides [2,7]. In the method used here, cartridges filled with octadecyl phase chemically bound to silica gel were applied for extraction of ROX and erythromycin. The presence of basic nitrogen(s) in macrolide molecules reflects the basic character of these lipophilic compounds; pK_a of roxithromycin is equal to 8.8. Therefore, most of the previous assays describe extraction of these substances from biological matrices at high pH. To obtain such conditions, 1 M NaOH [4,6],

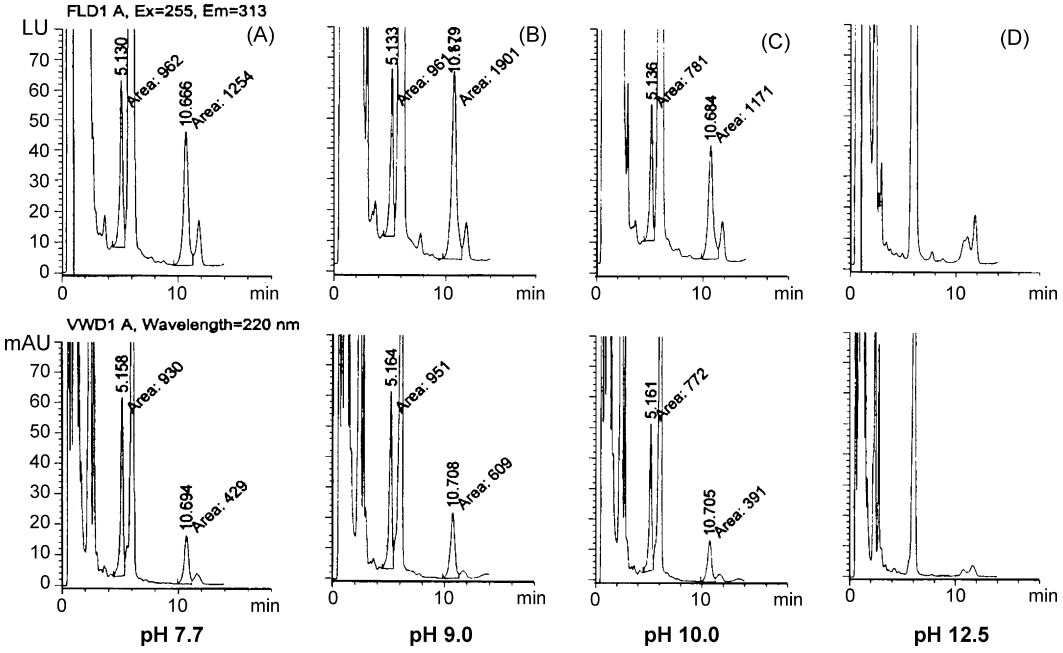

Fig. 1. HPLC chromatograms from the analysis of ROX and I.S. after SPE from human plasma at pH 7.7–12.5 (A–D). Erythromycin (5.1 min); ROX (10.7 min). Fluorescence detection (excitation wavelength of 255 nm, emission wavelength of 315 nm) (upper panel); UV detection (wavelength of 220 nm) (lower panel).

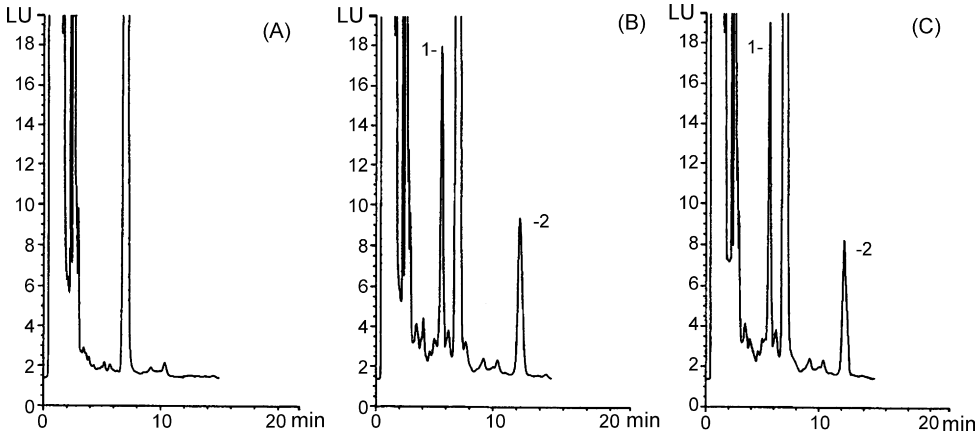

Fig. 2. HPLC chromatograms of ROX and I.S. obtained after SPE of 0.5 ml human plasma samples: (A) blank plasma; (B) plasma spiked with 10.0 mg/l of ROX and 20.0 mg/l of I.S.; (C) plasma sample of a healthy volunteer at 0.5 h elapsed from the administration of a single dose of 150 mg ROX tablet (8.9 mg/l of ROX). Peaks 1 and 2 denote I.S. and ROX, respectively.

buffers of pH ≥ 9 [2,7,8] or sodium carbonate solution of pH approximately 12 [3,9,10] have been used. However, under these conditions, many endogenous compounds in plasma were also extracted, so that in the present report, the influence of basic pH on recovery of ROX and I.S. from human plasma using SPE was studied to determine the optimal extraction conditions. The yields of ROX extraction obtained at pH 7.7, 9.0 and 10.0 were comparable. At pH 12.5, the peaks of ROX and erythromycin on the chromatogram were at the baseline level (Fig. 1). This could result from degradation of the macrolides or hydrolysis of the octadecyl phase-silica bond or both reactions simultaneously. So, to overcome problems with stability of sorbent with the octadecyl phase, which is stable in the pH range of 2–8, the optimal conditions for extraction of the analytes from plasma were established with the use of phosphate buffer of pH 7.5 and therefore, extraction at this pH value was finally used in the validated method. At this pH value, yield for SPE of I.S. amounted to 83.8 ± 2.8% and corresponded to literature data [4,6], but the absolute yield of ROX at concentrations of 1.5 and 10.0 mg/l, prepared from 6 and 12 samples, was 38.9 ± 3.5% and 43.1 ± 2.4%, respectively. ROX and erythromycin differ from each other in the side chain at position 9. In the ROX molecule, an additional basic nitrogen atom exists, providing the main reason for the difference in extraction using cartridges with C_{18} phase. In spite of its lower extraction yield, the analytical range of the calibration curve, especially LLOQ, was suitable for pharmacokinetic studies of ROX in human plasma.

Chromatograms obtained after extraction of blank plasma and the plasma samples containing ROX and I.S. are presented in Fig. 2. The peaks of the I.S. and ROX are well separated from each other and from the peak of another product of derivatization, FMOC-OH, with a retention time of 6.8 min. This compound probably formed as a result of hydrolysis of FMOC-Cl [10] and has not influenced the separation of ROX and I.S. Moreover, no peaks of endogenous compounds were found to interfere with the peaks of the analytes, as proved on the chromatograms (Fig. 2).

The standard curve for ROX was linear in the range of ROX concentration 0.5–10.0 mg/l. An established equation was used to calculate the plasma concentration in volunteers following administration of a tablet with 150 mg of ROX. The correlation coefficient r was calculated to confirm linearity of the calibration curve (Table 1).

For the calculation, LOD (S/N = 11:1) was 0.2 mg/l, while LLOQ amounted to 0.5 mg/l. The designed technique was characterized by high precision of estimation of ROX concentration both within the day and between days, as proved by CV ≤ 13% and accuracy expressed as percent error of estimates ≤12% (Table 1).

ROX proved to be stable after three freeze–thaw cycles, as demonstrated by CV ≤ 7.2% and percent error of estimates below 6%. The stability during storage in an autosampler for 6 h was expressed by high accuracy and precision with CV below 7%.

3.3. In vivo application—pharmacokinetic studies

This method was used for pharmacokinetic studies on ROX. The pharmacokinetic profile was followed for up to 48 h after

Table 1
Validation parameters of standard curve for analysis of ROX in human plasma[a]

Nominal concentration (mg/l)	Mean assayed value (mg/l)	Precision (CV, %)	Accuracy (%error)
Intra–day repeatability ($n = 6$)			
1.50	1.52	9.0	1.3
5.00	5.10	3.0	2.0
10.00	10.13	5.6	1.3
Inter–day reproducibility ($n = 7$)[b]			
0.50	0.56	9.1	12.0
0.75	0.78	11.7	4.0
1.50	1.33	5.2	−11.3
2.00	1.79	10.0	−10.5
5.00	4.58	12.6	−8.4
7.50	7.24	9.3	−3.5
10.00	10.44	9.4	4.4

[a] Equation of calibration curve for ROX: $y = 0.113 \times x - 0.019$ ($r = 0.997$).
[b] Seven calibration curves were prepared during 2 months period.

Table 2
Pharmacokinetic parameters of ROX obtained after single oral administration of 150 mg ROX tablets to healthy volunteers compare with literature data

Pharmacokinetic parameters	Present study (mean ± SD)	Literature data		
		150 mg [11]	300 mg [11]	300 mg [14]
$t_{0.5}$ (h)	6.0 ± 3.0	8.3	10.9	7.2 ± 2.5
C_{max} (mg/l)	6.0 ± 1.9	6.6	9.7	9.1 ± 2.1
		6.7 ± 2.6 [12]	11.0 ± 2.2 [12]	
t_{max} (h)	1.2 ± 1.1	1.6	1.3	nr
Vd (l)	24.2 ± 9.1	nr	nr	nr
Cl (ml/min)	50.1 ± 13.0	nr	nr	nr
$AUC_{0\rightarrow48}$	51.2 ± 14.4	nr	nr	nr
$AUC_{48\rightarrow\infty}$	2.6 ± 6.4	nr	nr	nr
$AUC_{0\rightarrow\infty}$ (mg h l^{-1})	53.8 ± 17.4	69.4	98.6	116.9 ± 32.7

nr: not reported.

oral administration of a tablet with 150 mg of ROX. The drug was rapidly absorbed from the gastrointestinal tract and the plasma level of ROX reached C_{max} = 6.0 ± 1.9 mg/l at t_{max} = 1.2 ± 1.1 h (Table 2). The values of ROX concentration in plasma agree with previously published reports [1,11,12]. Moreover, the ROX levels were greater than those of other macrolides, such as erythromycin [5], azithromycin and clarithromycin [13], administered at higher doses. After administration of the 150 mg tablet, $t_{0.5}$ of ROX amounted to 6 h (Table 2 and [11]), whereas some previous data after a higher, 300 mg dose, reported $t_{0.5}$ of 9.7–19 h [11,13]. The long half-life of ROX can be an advantage over other macrolides due to the need for less frequent dosing.

4. Conclusion

HPLC analysis proved that fluorescence derivatives of ROX and erythromycin possess different ultraviolet and fluorescence activities. Therefore, application of fluorescence detection for estimation of ROX is sound, since it results in a three-fold augmentation of ROX detectability, compared to that using UV detection. However, in the case of erythromycin, the two detectors can be used interchangeably, since the choice does not affect detectability of the analyte in plasma. The procedure in comparison to others reported in literature [1–4] is completely validated including stability test and the application of the method to in vivo conditions. Moreover, this technique enables the determination of lower levels of ROX (LLOQ of 0.5 mg/l) and therefore it is more useful in pharmacokinetic studies after administration of low doses of the drug than the method discussed in [10].

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