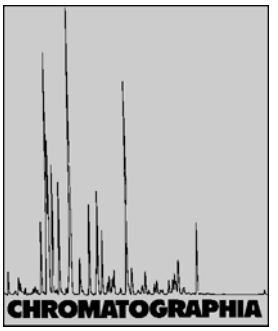
Isocratic Liquid Chromatographic Method for the Analysis of Roxithromycin and Structurally Related Substances in Bulk Samples



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Key Words

Column liquid chromatography
Isocratic elution
Roxithromycin and homologs
Bulk sample analysis

Summary

A selective, repeatable, and robust isocratic liquid chromatographic method has been developed for the analysis of bulk samples of roxithromycin, a semi-synthetic macrolide antibiotic. The method involves the use of XTerra RP18 as stationary phase and acetonitrile-2.0 M $(NH_4)_2HPO_4$, pH 6.4-water, 25:30:45, (*v/v*) as mobile phase, delivered at a flow rate of 1.0 mL min^{-1}. Detection was by UV absorbance at 215 nm. Sufficient separation of roxithromycin from its homolog containing one more methyleneoxy group (roxithromycin G) and from other related substances was achieved. The robustness of the method was evaluated by means of a full-factorial experimental design.

Introduction

Roxithromycin is a semi-synthetic 14-membered macrolide antibiotic in which the 9-keto group of the erythromycin A (EA) is substituted by an ether-oxime chain [1]. The *E* isomer is more active than the *Z*. Figure 1 shows the structure of roxithromycin and its related substances and degradation products, and the abbreviations used throughout this manuscript.

Liquid chromatographic (LC) methods have been reported for the analysis of roxithromycin (RXT) in biological samples, with either coulometric or electrochemical detection [2–4]. These modes of detection were used mainly to improve the sensitivity because roxithromycin has a very weak ultraviolet chromophore. The European Pharmacopoeia (Ph. Eur.) prescribes a gradient LC method with UV detection at 205 nm for the related substances test and assay of roxithromycin [5]. Two mobile phases were used, 31.5:20:48.5 (*v/v*) acetonitrile-1.48 M ammonium phosphate, pH 5.3-water and 70:30 (*v/v*) acetonitrile-water. This method suffers from a negative gradient slope as elution progresses, because of changes in the buffer concentration. More important, the roxithromycin homolog containing one more methyleneoxy group (RXT G) is poorly separated from the main component (RXT). An LC method employing Supelcosil LC C18 ABZ +Plus at 30 °C with a pH 4.3 mobile phase was found to be more suitable for the determination of RXT and its related substances [6]. Although this was the first method which partially separated RXT G from RXT, rather complex gradient elution was needed. It was, therefore, decided to develop a simpler method that was at least as selective.

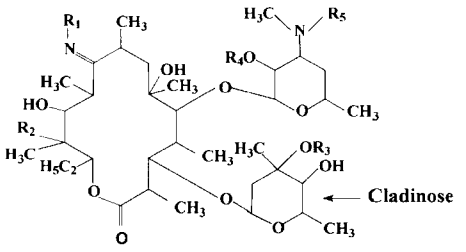
XTerra RP18 has recently been used successfully in this laboratory as a stationary phase for the determination of erythromycins in fermentation broth, erythromycin in bulk samples, and troleandomycin bulk sample [7–9]. Good selectivity and sensitivity were obtained. XTerra RP 18 is a recent addition to reversed-phase adsorbents and is based on silica gel on to which methyl groups have been incorporated. Its organosilane substituent contains a polar carbamate group, to prevent polar components of the basic analyte from interacting with the free silanol groups on the surface of the silica gel. It is claimed to have extended pH stability, to be thermally more stable, and to be more efficient than classical silica-based packings.

Experimental

Reagents and Samples

Acetonitrile, HPLC grade S, was from Biosolve (Valkenswaard, Netherlands). Diammonium hydrogen phosphate (Acros Organics, Geel, Belgium) 2.0 M solution was adjusted to the required pH by

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	R_1	R_2	R_3	R_4	R_5	Clad
Roxithromycin A (RXT)	$CH_3O(CH_2)_2OCH_2O$	OH	CH_3	H	CH_3	+
Decladinosylroxithromycin (RXT B)	$CH_3O(CH_2)_2OCH_2O$	OH	-	H	CH_3	-
Erythromycin A oxime (E-isomer) (RXT C)	H	OH	CH_3	H	CH_3	+
N-demethylroxithromycin (RXT F)	$CH_3O(CH_2)_2OCH_2O$	OH	CH_3	H	H	+
Roxithromycin Z-isomer (RXT D)	$OCH_2O(CH_2)_2OCH_3$	OH	CH_3	H	CH_3	+
Roxithromycin homologue (RXT G)	$CH_3O(CH_2)_2OCH_2OCH_2O$	OH	CH_3	H	CH_3	+
Roxithromycin B (RXT H)	$CH_3O(CH_2)_2OCH_2O$	H	CH_3	H	CH_3	+
Roxithromycin I (RXT I)	$CH_3O(CH_2)_2OCH_2O$	OH	CH_3	$CH_3O(CH_2)_2OCH_2O$	CH_3	+

Figure 1. The structures of roxithromycin and related substances.

addition of 2.0 M phosphoric acid (Merck, Darmstadt, Germany). Dipotassium and disodium hydrogen phosphates were from Merck. Deionized water was distilled from glass apparatus.

Roxithromycin bulk samples and roxithromycin related reference substances – decladinosylroxithromycin (RXT B), erythromycin A oxime *E* isomer (RXT C), roxithromycin *Z* isomer (RXT D), *N*-demethylroxithromycin (RXT F), roxithromycin homolog containing one more methyleneoxy group (RXT G), roxithromycin B (RXT H), and roxithromycin I (RXT I) were obtained from Roussel Uclaf, France. Roxithromycin A (RXT) reference substance (CRS, 96.1%) and erythromycin A (EA) reference substance (CRS) were obtained from the Ph. Eur.

LC Instrumentation and Chromatographic Conditions

LC was performed with a SpectraSystem P1000XR quaternary pump, a SpectraSeries AS100 Autosampler equipped with a 100-µL loop, a Spectra 100 UV-visible variable-wavelength detector set at 215 nm (Thermo Separation Products, Fremont, CA, USA), and a Hewlett-Packard (Avondale, PA, USA) model HP 3396 series II integrator. XTerra RP18, 5 µm (25 cm × 4.6 mm i. d. column; Waters, Milford, MA, USA) was used as stationary phase at 35 °C. The temperature was controlled by means of a thermostat (Julabo, Seelsbach, Germany) immersed in a water-bath. Acetonitrile-2.0 M ammonium phosphate, pH 6.4-water, 25:30:45 (*v*/*v*), delivered at 1.0 mL min^{-1}, was used as mobile phase. The mixture was degassed by purging with helium. The injection volume was 100 µL.

Sample Preparation

For identification purposes, solutions of the related substances were prepared separately at a concentration of 0.1 mg mL^{-1}. A test mixture containing all the compounds mentioned above, at a concentration of 0.1 mg mL^{-1}, was also prepared. This solution was used in method development and in the robustness study. EA was not included because it is not present in roxithromycin commercial grade samples, and if it is present it is separated from other polar components, because it is eluted very early (7.5 min). Acetonitrile-water, 3:7 (*v*/*v*) was used as dissolution solvent.

Software

DryLab software 2000 (LC Resources, Berlin, Germany) was used to determine the optimum column temperature and mobile-phase pH for the separation. Set-up of the full-factorial design applied, and analysis of the measured response variables and multivariate regression calculation, was supported by the statistical graphics software system Statgraphics plus, standard edition version 4.1 (Manugistics, Rockville, MD, USA).

Results and Discussion

Method Development

Initial studies using XTerra RP18 as stationary phase, at 65 °C, and acetonitrile-0.2 M K_2HPO_4, pH 7.0-water, 35:5:60 (*v*/*v*) as mobile phase (conditions adopted from earlier work on the separation of erythromycins in bulk samples [7, 8]) resulted in good separation of all components except RXT G, which coeluted with RXT. The total analysis time was well beyond 2 h. Experiments to verify further the separation of roxithromycin and its related substances were performed at a lower temperature (30 °C) with the same mobile phase. This was because elution of macrolide antibiotics is more rapid at lower temperatures, because they are better solvated and thus interact less with the apolar stationary phase. (This chromatographic retention behavior of macrolides had been reported also for erythromycin and spiramycin [8, 10, 11].) Separation of the critical pair RXT and RXT G was given special emphasis. Again, good separation was obtained for all components, except for the critical pair mentioned above. It was clear, therefore, that further investigation of the type of buffer and buffer concentration, mobile phase pH, acetonitrile concentration, and temperature were necessary to achieve optimum separation of all the components, including these two homologs.

The use of 5 to 25% sodium, potassium, or ammonium phosphates, concentration 0.2 M, at pH 7.0 with acetonitrile (35%) at 30 °C was investigated. Although separation of the critical pair was not achieved, when the ammonium phosphate concentration was 25% the RXT peak was distorted by a shoulder. Ammonium phosphate buffer was, therefore, chosen for further experimental work.

The effect on the separation of the use of 10 to 30% (*v*/*v*) ammonium phosphate buffer, pH 7.0, at concentrations between 1.5 and 2.5 M, was investigated. A low acetonitrile content (26% *v*/*v*) was used because of buffer-solubility problems. Better separation of all the components, includ-

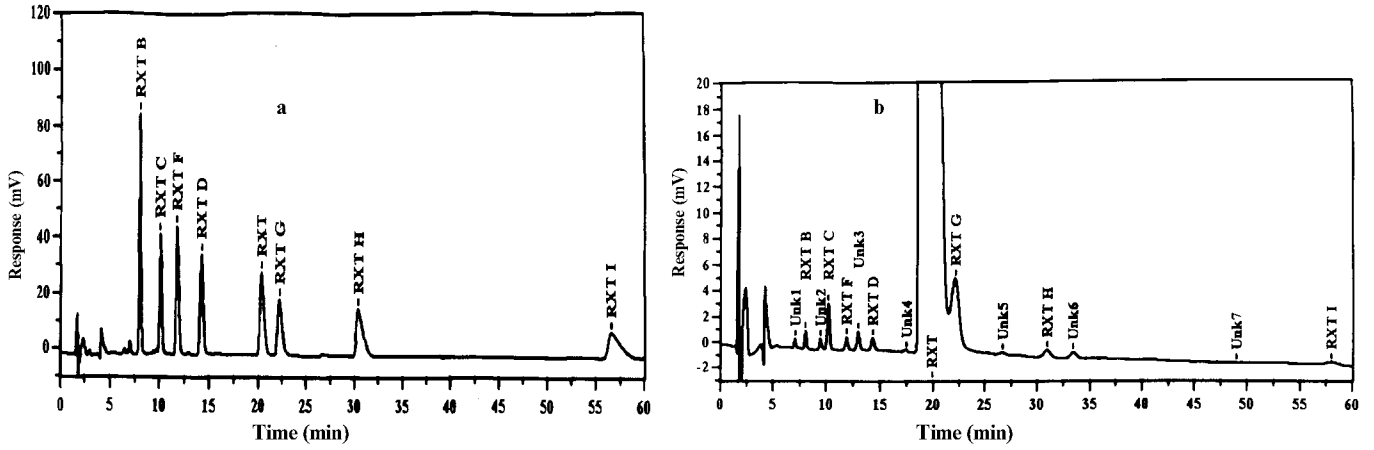


Figure 2. Typical chromatograms obtained from (**a**) a test mixture of roxithromycin reference substances (concentration 0.1 mg mL^{-1}) and (**b**) a roxithromycin bulk sample (concentration = 2.0 mg mL^{-1}). Column 25 cm × 4.6 mm i. d., 5 µm particle, XTerra RP18, at 30 °C. Mobile phase 25:30:45 (*v/v*) acetonitrile-2.0 M $(NH_4)_2HPO_4$, pH 6.4-water. Flow rate 1.0 mL min^{-1}. Volume injected 100 µL (total mass injected: (**a**) 10 µg, (**b**) 200 µg). UV detection set at 215 nm. Unk = Unknown.

ing baseline separation of RXT and RXT G, was obtained by use of 25% (*v/v*) 2.5 M buffer, but use of this mobile phase led to problems of salt precipitation. An intermediate buffer concentration of 2.0 M (30% *v/v*) was chosen as a compromise for further experimental work, because it furnished optimum separation. The column back pressure was approximately 140 bar and the total analysis time was ca 1 h. Because of buffer-solubility problems the maximum acetonitrile content was fixed at 25% (*v/v*).

Temperature and mobile phase pH were optimized by use of the DryLab software with acetonitrile-2.0 M ammonium phosphate, pH *X*-water 25:30:45 (*v/v*) as mobile phase. The effect of temperature was examined at 30 °C and 40 °C and mobile phase pH investigated was 6.0, 6.5 and 7.0. Because a combination of 35 °C and pH 6.4 mobile phase resulted in baseline separation of all the components in the test mixture, the mobile phase finally chosen was 25:30:45 (*v/v*) acetonitrile-2.0 M $(NH_4)_2HPO_4$, pH 6.4-water. Chromatograms obtained from a test mixture and from a 2.0 mg mL^{-1} solution of a commercial bulk sample are shown in Figures 2a and b, respectively.

Robustness

Robustness is an important aspect of method validation. It entails evaluation of the effects of small changes in the operating conditions (variables) of the analytical procedure on measured or calculated responses. The changes introduced when performing a robustness test reflect the changes that can occur when a method is transferred between different laboratories.

Table I. Robustness study, nominal values corresponding to low, central, and upper levels.

Chromatographic variable	Low value	Central value	High value
A, pH	6.2	6.4	6.6
B, Acetonitrile concentration (ACN,%)	24	25	26
C, Temperature (°C)	30	35	40

A three-factorial design experiment, with the help of the statistical graphic software system Statgraphics plus, was used to evaluate the robustness of the method. The chromatographic variables investigated were the concentration of organic modifier in the mobile phase, mobile phase pH, column temperature, and the concentration of phosphate buffer. The values of the variables in the design are listed in Table I. For these four variables, 2^3 different experiments, one central level experiment plus duplicate experiments, were performed and eighteen chromatograms obtained. The measured response variables were the retention times of the different erythromycin components. For each experiment, the average result from two analyses was used for further calculations. The estimated effects of the four variables, with their second-order interactions, on the selectivity between pairs RXT B and RXT C, RXT C and RXT F, RXT F and RXT D, RXT D and RXT, RXT and RXT G, RXT G and RXT H, and RXT H and RXT I are presented in the form of standardized pareto charts in Figure 3. The bars are displayed in order of the sizes of the effects, with the largest effects at the top. The charts include a vertical line at the critical *t*-value for an α of 0.05. Effects for which the bars are smaller than the critical *t*-value are regarded as not significant.

The charts, under the examined conditions, clearly show that increasing the pH has a positive influence on the selectivities for the pairs RXT B and RXT C, RXT F and RXT D, RXT D and RXT, and RXT G and RXT H, but negatively affects the selectivities for RXT C and RXT F, RXT and RXT G, and RXT H and RXT I. Acetonitrile content (ACN, %) is the second most important condition affecting the selectivity. It has a negative effect on selectivity for RXT B and RXT C, RXT C and RXT F, RXT D and RXT, RXT and RXT G, RXT G and RXT H, and RXT H and RXT I, i. e. the selectivity of the separation of these pairs is usually reduced when the ACN concentration is increased. Increasing ACN (%) does, however, positively affect the selectivity for RXT F and RXT D. Increasing the column temperature positively influences the selectivity for RXT F and RXT D, and RXT D and RXT and has a negative effect on that for RXT H and RXT I. Second-order interactions between the chromatographic conditions has a moderate effect only on selectivity, with AB and AC being important.

Because pH and ACN (%) have the largest effects on selectivity, response surface

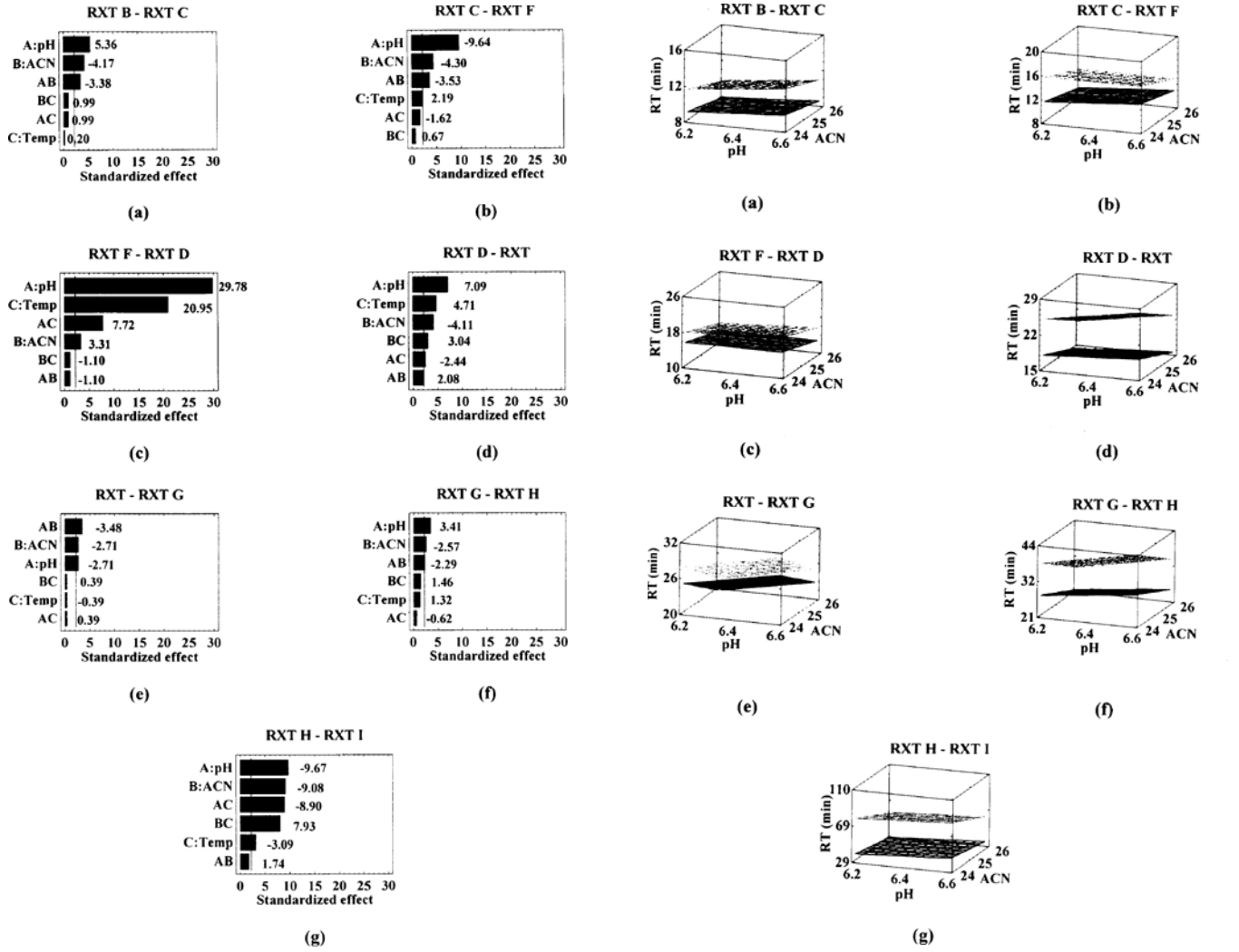


Figure 3. Standardized pareto charts representing the estimated effects of chromatographic conditions (A, B, C) and their interactions (AB, BC, and AC) on the selectivity for (**a**) RXT B and RXT C, (**b**) RXT C and RXT F, (**c**) RXT F and RXT D, (**d**) RXT D and RXT, (**e**) RXT and RXT G, (**f**) RXT G and RXT H, and (**g**) RXT H and RXT I. A is mobile phase pH, B is the concentration of acetonitrile (%), and C is the column temperature (°C).

Figure 4. Estimated response plots for (**a**) RXT B and RXT C, (**b**) RXT C and RXT F, (**c**) RXT F and RXT D, (**d**) RXT D and RXT, (**e**) RXT and RXT G, (**f**) RXT G and RXT H, (**g**) RXT H and RXT I (the lower and upper planes correspond to the first and second components, respectively, of each pair) constructed with the retention times as a function of pH and acetonitrile concentration (%).

plots were constructed using these two variables. Figure 4 shows how the retention times (RT, min) for RXT B, RXT C, RXT F, RXT D, RXT, RXT G, RXT H, and RXT I change as pH and ACN (%) are altered. The plots clearly show that the peaks are always separated in the range of conditions examined, because the planes do not overlap. This means the method is robust within the range of conditions examined.

Repeatability, Linearity, and Detection Limits

The repeatability of the method was assessed by sixfold injection of a 2.0 mg mL^{-1} solution of RXT. The relative standard deviation (*RSD*) was 0.2%. The linearity was determined by analysis (n = 3) of solutions of RXT corresponding to 0.1%, 1%, 5%, 50%, 75%, 100%, and 125% of the sample concentration in a test solution (2.0 mg mL^{-1}). The relationships obtained were y = 1651720 x – 8977, r = 0.9999, and $S_{y,x}$ = 59751, where y is the peak area, x the concentration in mg mL^{-1}, r the correlation coefficient, and $S_{y,x}$ the standard error of estimate. For injection of 200 µg the limit of quantitation (*LOQ*) with a signal-to-noise ratio of 10 was: 0.04% for RXT B (*RSD* = 19.6%, n = 6) and RXT C (*RSD* = 18.6%, n = 6); 0.05% for RXT F (*RSD* = 18.4%, n = 6), RXT D (*RSD* = 17.2, n = 6), and RXT (*RSD* = 10.9%, n = 6); 0.06% for RXT G (*RSD* = 21.0%, n = 6) and RXT H (*RSD* = 11.2%, n = 6); and 0.15% for RXT I (*RSD* = 11.1%, n = 6). The limit of detection (*LOD*) with a signal-to-noise ratio of 3 was: 0.01% for RXT B and RXT C; 0.015% for RXT F, RXT D, and RXT; 0.02% for RXT G and RXT H and 0.05% for RXT I.

Analysis of Commercial Samples

Four bulk samples of roxithromycin from a manufacturer were analyzed using the described method. The results obtained for the samples are presented in Table II. Two different solutions were each analyzed three times. The roxithromycin (RXT) content (% *m/m*) was calculated by comparison with the European Pharma-

Table II. Composition (%, *m/m*), with *RSD* values (%, $n = 6$) in parentheses, of roxithromycin bulk samples determined by chromatography on 5 µm XTerra RP_{18} (25 cm × 4.6 mm i. d. column).

Identity	*RRT*	Sample			
		1	2	3	4
Unk 1	0.38	0.1 (31)	0.05	ND	ND
RXT B	0.42	ND	0.2 (29)	ND	ND
Unk 2	0.48	ND	ND	0.1 (16)	0.1 (28)
RXT C	0.52	0.04*	0.3 (3.0)	0.04*	0.03*
RXT F	0.60	ND	0.1 (14)	0.1 (9.4)	0.1 (10)
Unk 3	0.67	0.04*	0.1 (3.5)	0.2 (3.5)	0.2 (4.0)
RXT D	0.72	0.03*	0.1 (6.2)	0.04*	0.04*
Unk 4	0.85	ND	0.03*	ND	ND
RXT	1.00	97 (0.1)	96 (0.3)	96 (0.5)	97 (0.8)
RXT G	1.15	0.05	0.7 (2.9)	0.1 (16)	0.2 (6.6)
Unk 5	1.35	ND	0.04*	0.1 (19)	0.04*
RXT H	1.58	ND	0.1 (5.7)	0.2 (3.8)	0.2 (3.2)
Unk 6	1.70	0.2 (4.1)	0.1 (3.2)	0.4 (3.3)	0.4 (2.0)
Unk 7	2.63	ND	ND	0.8 (5.0)	0.8 (3.7)
RXT I	2.75	ND	0.1 (20)	0.1 (14)	0.2 (13)
Water		NP	2.3 (5.5)	2.4 (3.8)	2.3 (4.1)
Total		97.4	100.1	100.5	101.5

* *RSD* values were not calculated because they were below the *LOQ; RRT* = Retention time relative to that of RXT; Unk = Unknown; ND = below the *LOD*; NP = Not performed because the amount of sample was insufficient.

copoeia Chemical Reference Substance (Ph. Eur. CRS) with an assigned purity of 96.1%. The mean content of the main component in all samples was >95% with low *RSD* values. The content (% *m/m*) of related substances and other unknown impurities was calculated by comparison with a 0.5% dilution of the Ph. Eur. CRS solution; no relative response factors were used. Several of the unknown impurities exceed 0.1%. The water content was determined by Karl Fischer titration. The total content of roxithromycins was approximately 100% in all samples except sample 1, for which the water content was not determined because the amount of sample was insufficient.

Conclusion

It can be concluded that a simple isocratic LC method with XTerra RP_{18} as stationary phase is suitable for the determination of roxithromycin and its related substances. This method is selective, sensitive, linear, repeatable, and robust.

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