Journal of Chromatography B, 877 (2009) 1771–1777

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Simultaneous determination of clarithromycin, rifampicin and their main metabolites in human plasma by liquid chromatography–tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 13 February 2009
Accepted 28 April 2009
Available online 3 May 2009

Keywords:
LC/MS/MS
Clarithromycin
Rifampicin
TDM
Pharmacokinetics

ABSTRACT

The drug combination rifampicin and clarithromycin is used in regimens for infections caused by Mycobacteria. Rifampicin is a CYP3A4 inducer while clarithromycin is known to inhibit CYP3A4. During combined therapy rifampicin concentrations may increase and clarithromycin concentrations may decrease. Therefore a simple, rapid and easy method for the measurement of the blood concentrations of these drugs and their main metabolites (14-hydroxyclarithromycin and 25-desacetylrifampicin) is developed to evaluate the effect of the drug interaction. The method is based on the precipitation of proteins in human serum with precipitation reagent containing the internal standard (cyanoimipramine) and subsequently high-performance liquid chromatography (HPLC) analysis and tandem mass spectrometry (MS/MS) detection in an electron positive mode. The method validation included selectivity, linearity, accuracy, precision, dilution integrity, recovery and stability according to the "Guidance for Industry – Bioanalytical Method Validation" of the FDA. The calibration curves were linear in the range of 0.10–10.0 mg/L for clarithromycin and 14-hydroxyclarithromycin and 0.20–5.0 mg/L for rifampicin and 25-desacetylrifampicin, with within-run and between-run precisions (CVs) in the range of 0% to −10%. The components in human plasma are stable after freeze–thaw (three cycles), in the autosampler (3 days), in the refrigerator (3 days) and at room temperature (clarithromycin and 14-hydroxyclarithromycin: 3 days; rifampicin and 25-desacetylrifampicin: 1 day). The developed rapid and fully validated liquid chromatography–tandem mass spectrometry (LC/MS/MS) method is suitable for the determination of clarithromycin, 14-hydroxyclarithromycin, rifampicin and 25-desacetylrifampicin in human plasma.

1. Introduction

Rifampicin (rifampin) is combined with clarithromycin to improve efficacy and prevent resistance in drug regimens for the treatment of Mycobacterial infections [1].

Clarithromycin is primarily metabolised by cytochrome P450 (CYP) 3A iso-enzymes to an active 14-hydroxy metabolite [2] and rifampicin to the active metabolite 25-desacetylrifampicin [3]. Rifampicin is known to induce several metabolic liver enzymes, including CYP P450 iso-enzymes (e.g. CYP1A2, 2C9, 2C19, and 3A4) [4], while clarithromycin is an inhibitor of CYP3A4 [2].

Several small studies have suggested that rifampicin may decrease the clarithromycin serum concentrations without influencing the 14-hydroxyclarithromycin levels [5,6]. The effect of clarithromycin on the metabolism of rifampicin and its plasma levels is unknown.

In a randomised controlled trial in Ghana [BURULICO; NCT00321178] the standard treatment for *Mycobacterium ulcerans* infection (streptomycin and rifampicin for 8 weeks) is compared with streptomycin and rifampicin for 4 weeks followed by clarithromycin and rifampicin for 4 weeks. The pharmacokinetics of clarithromycin and rifampicin are studied in a subset of patients to evaluate the effect of the drug interaction on the plasma concentration of the drugs.

Therefore a simple, rapid and easy method for the measurement of the plasma concentrations of these drugs and their metabolites had to be set up.

Several HPLC-based methods for the detection of the individual drugs have been developed, but no previous method with a simultaneous determination of the two drugs and their main metabolites has been described. Chromatographic methods with fluorescent, ultraviolet, electrochemical or amperometric detection of the individual drugs [7–15] are time consuming, mostly require large sample volumes and are less sensitive

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doi:10.1016/j.jchromb.2009.04.038

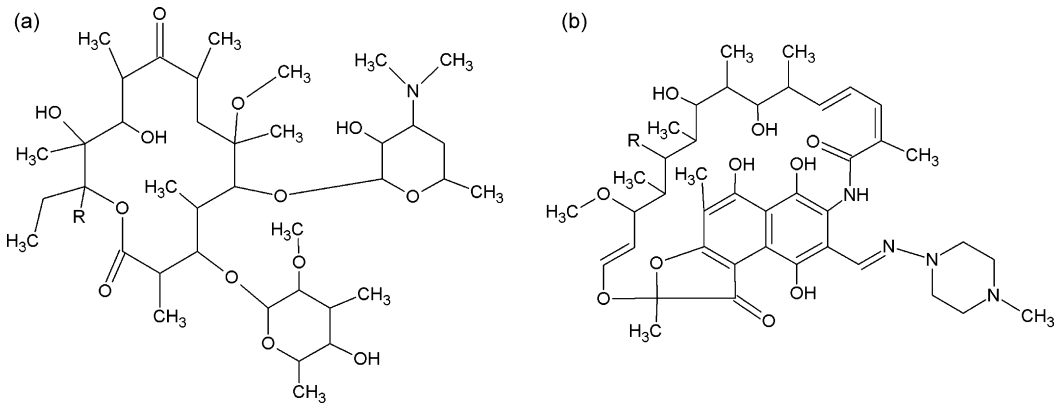

Fig. 1. (a) Chemical structure of clarithromyin (R=H) and 14-hydroxy-clarithromycin (R=OH). (b) Chemical structure of rifampicin (R=OH) and 25-desacetyl-rifampicin (R=$COOCH_3$).

than methods with mass spectrometric detection. The reported chromatographic methods for clarithromycin with tandem mass spectrometric detection (LC/MS/MS) [16–19] achieve sufficient sensitivities in short run times, but suffered from lacking the active 14-hydroxymetabolite. In one LC/MS/MS method the 14-hydroxyclarithromycin concentration was only estimated [20]. Two previous LC/MS/MS methods for the determination of rifampicin and the 25-desacetylmetabolite have been reported. The first method [21] determined the components in plasma and used a normal phase column. The other method [22], determining the components in serum, was limited by methodological flaws (i.e. the purity of the components and the linearity of the calibration curves). Two methods, describing the determination of rifampicin in plasma [23] or tissue [24] lacked measuring the metabolite of rifampicin.

For therapeutic drug monitoring, many drugs are measured in plasma or serum and are analyzed with a reversed phase column. Standardization of these chromatographic and mass spectrometric characteristics improves the efficiency, flexibility and cost effectiveness of therapeutic drug monitoring.

This paper describes a simple, rapid and fully validated LC/MS/MS method for the determination of clarithromycin, rifampicin and their main metabolites in human plasma, using a reversed phase column and electrospray positive ionization.

2. Experimental

2.1. Reagents

Clarithromycin ($C_{38}H_{69}NO_{13}$) and 14-hydroxyclarithromycin ($C_{38}H_{69}NO_{14}$) were provided by Abbott (IL, USA). Rifampicin ($C_{43}H_{58}N_4O_{12}$) and 25-desacetylrifampicin ($C_{41}H_{56}N_4O_{11}$) were provided by Sanofi-Aventis (Frankfurt, Germany). The chemical structures of these components are shown in Fig. 1.

The internal standard, cyanoimipramine, was supplied by Roche (Woerden, The Netherlands).

Acetonitrile for LC/MS and water for LC/MS were purchased from BioSolve BV (Valkenswaard, The Netherlands). Methanol Lichrosolv and the other used chemicals were of HPLC or analytical grade and were obtained from VWR (Amsterdam, The Netherlands).

The precipitation reagent consisted of 0.04 mg/L cyanoimipramine dissolved in a mixture of methanol and acetonitrile (4:21, v/v).

Pooled human plasma samples with EDTA as anticoagulant were put available according to the rules of the University Medical Center Groningen.

2.2. Calibration standards and quality control samples

Separate stock solutions for the calibration standards and QC samples were prepared by dissolving the components in methanol/water (1:1, v/v). These stock solutions were diluted with methanol/water (1:1, v/v) to prepare working stock solutions.

The calibration standards and QC samples were prepared by diluting the stock or working stock solution with controlled pooled human plasma. The amount of the methanol solutions added to the plasma was less than 5% of the final volume.

The calibration standards and QC samples were prepared on day 0 and stored at −20 °C.

The concentrations of the stock solutions, calibration standards and QC samples are listed in Table 1.

2.3. Sample preparation

Frozen samples were thawed at room temperature. Of each sample (blank plasma, calibration standard or QC sample) 10 μL and 750 μL precipitation reagent containing the internal standard were vortexed for 1 min, stored at −20 °C for about 30 min to promote protein precipitation and thereafter centrifuged at 11,000 × *g* for 5 min. From the clear upper layer 5 μL was injected onto the LC/MS/MS system.

2.4. LC/MS/MS conditions

All experiments were performed on a Thermo Fisher (San Jose, USA) triple quadrupole LC/MS/MS with a Finnigan™ Surveyor® LC

Table 1
Concentrations (mg/L) of stock solutions, calibration standards and QC samples.

Component	Stock solution	Working stock solution	Calibration standards	QC samples			
				LLOQ	LOW	MED	HIGH
Clarithromycin	500	50	0.10, 0.50, 1.0, 2.0, 4.0, 7.0, 10.0	0.10	0.20	5.0	8.0
14-Hydroxyclarithromycin	500	50	0.10, 0.50, 1.0, 2.0, 4.0, 7.0, 10.0	0.10	0.20	5.0	8.0
Rifampicin	250	50	0.20, 0.50, 1.2, 2.0, 3.0, 4.0, 5.0	0.20	1.0	2.5	4.5
25-Desacetylrifampicin	250	50	0.20, 0.50, 1.2, 2.0, 3.0, 4.0, 5.0	0.20	1.0	2.5	4.5

Table 2
LC/MS/MS conditions.

Component	Parent $[M-H]^+$ (m/z)	Product $[M-H]^+$ (m/z)	Collision energy (eV)
Clarithromycin	748.5	590.2	18
14-Hydroxyclarithromycin	764.4	606.2	20
Rifampicin	823.3	791.2	17
25-Desacetylrifampicin	781.4	749.2	14
Cyanoimipramine	306.2	218.0	39

pump and a Finnigan™ Surveyor® autosampler which was set at a temperature of 20 °C. After sample preparation, 5 μL of the clear upper layer was injected on a 50 mm × 2.1 mm C_{18}, 5-μm analytic column (HyPurity Aquastar, Interscience Breda, The Netherlands) for chromatographic separation. The column temperature was set at 20 °C. The mobile phase had a flow of 0.3 mL/min and consisted of an aqueous buffer (containing ammonium acetate 10 g/L, acetic acid 35 mg/L and trifluoroacetic anhydride 2 mL/L water), water and acetonitrile. The mobile phase operated in the following gradient: 0–2 min: 5% buffer, 95% at 0 min to 0% at 2 min water and 0% at 0 min to 95% at 2 min acetonitrile; 2–3 min: 5% buffer, 0% water and 95% acetonitrile; 3–3.1 min: 5% buffer, 0% at 3 min to 95% at 3.1 min water and 95% at 3 min to 0% at 3.1 min acetonitrile; 3.1–3.6 min: 5% buffer, 95% water and 0% acetonitrile.

The Finnigan™ TSQ® Quantum Discovery mass selective detector was operating in electrospray positive ionization mode and performed selected reaction monitoring (SRM) as scanning mode. The mass parameters were used at a scan width of 0.5 m/z and are listed in Table 2. The ion source spray voltage was set at 3500 V, the sheath and auxiliary gas pressure at 35 Arbitrary units (Arb.) and 5 Arb., respectively and the capillary temperature at 350 °C.

Xcalibur® software version 1.4 SR1 was used for peak height integration for all components.

2.5. Method validation

In accordance with the "Guidance for Industry – Bioanalytical Method Validation" of the FDA [25], method validation included selectivity, linearity, accuracy, precision and stability. Therefore, during 3 days, on each day a single calibration curve was obtained and the quality control samples were analyzed in fivefold. Linear regression was used to obtain calibration curves of the standards. Selectivity was evaluated by processing and analyzing six pools of blank human plasma.

Matrix effects (suppression or enhancement of ionization) were determined by continuous post-column infusion of a solution with clarithromycin, 14-hydroxyclarithromycin, rifampicin, 25-desacetylrifampicin and cyanoimipramine in methanol/water (1:1, v/v) and injection onto the column of first, the precipitation reagent without the internal standard, and second, processed blank plasma samples (precipitated with the precipitation reagent without the internal standard). Suppression or enhancement of the LC/MS/MS responses was investigated by comparing the responses of the blank precipitation reagent with the corresponding responses of the processed blank plasma samples.

The dilution integrity was investigated by diluting an over-the-curve QC (clarithromycin, 14-hydroxyclarithromycin: 25 mg/L; rifampicin, 25-desacetylrifampicin: 30 mg/L). Five over-the-curve QC's in 3 days were processed after a 10-fold dilution with blank human plasma.

Recoveries of the two drugs and the two metabolites were determined on three levels (LOW, MED, and HIGH) by comparing the average peak height of the components in the processed QC's in plasma with the average peak height of the components in the processed standard solutions in 50% methanol, representing 100% recovery. The recovery of the internal standard was calculated by dividing the average peak height of the internal standard in 20 processed plasma QC's by the average peak height of the internal standard in 20 blank precipitation reagent samples and multiplying by 100.

Stability tests including three freeze–thaw cycles, storage in the refrigerator (5 °C), storage at room temperature (20–25 °C) and storage in the autosampler (20 °C, re-injection stability of processed samples) were evaluated by QC samples on two levels (LOW and HIGH) in fivefold.

All calibration standards and QC samples were stored with protection to light, because rifampicin is known to be light-sensitive [23]. Besides, QC samples on two levels (LOW and HIGH) in fivefold were stored at room temperature without protection to light, to compare the results with the corresponding light-protected QC samples.

All samples (plasma samples and solutions in 50% methanol) were processed as described in Section 2.3.

3. Results

3.1. Chromatography

Cyanoimipramine, clarithromycin, 14-hydroxyclarithromycin, rifampicin and 25-desacetylrifampicin had a mean retention time of 2.50 min, 2.55 min, 2.25 min, 2.45 min and 2.30 min, respectively. An example of the chromatographic results is shown in Fig. 2.

3.2. Selectivity

The selectivity of this method was evaluated by analyzing six lots of pooled human plasma in comparison with LLOQ samples. There were no peaks observed in any of the pooled human plasma samples at the retention times of clarithromycin, 14-hydroxyclarithromycin, rifampicin, 25-desacetylrifampicin and cyanoimipramine.

3.3. Matrix effects

No suppression or enhancement was observed during the continuous post-column infusion.

3.4. Linearity

Over a range of 0.10–10.0 mg/L (clarithromycin, 14-hydroxyclarithromycin) the calibration curves were linear by using a weight factor of $1/x$. Over a range of 0.20–5.0 mg/L (rifampicin, 25-desacetylrifampicin) the calibration curves were linear (without using a weight factor). The interassay variability of the calibration curves is shown in Table 3.

3.5. Accuracy, precision and dilution integrity

The accuracy, within-run precision and between-run precision of the four QC's and the over-the-curve QC met the acceptance criteria suggested by the FDA (for LLOQ: <20% CV and <20% bias; for the other levels: <15% CV and <15% bias). The results were statistically analyzed by using one-way ANOVA. The data are listed in Table 4.

Table 3
Interassay variability of the calibration curves.

Component	Slope ± SD	Intercept ± SD	Correlation coefficient
Clarithromycin	0.283 ± 0.00236	0.000173 ± 0.00310	0.99934
14-Hydroxyclarithromycin	0.267 ± 0.00526	0.00142 ± 0.00703	0.99634
Rifampicin	0.137 ± 0.00442	0.00143 ± 0.0122	0.99027
25-Desacetylrifampicin	0.0532 ± 0.00147	−0.000191 ± 0.00409	0.99283

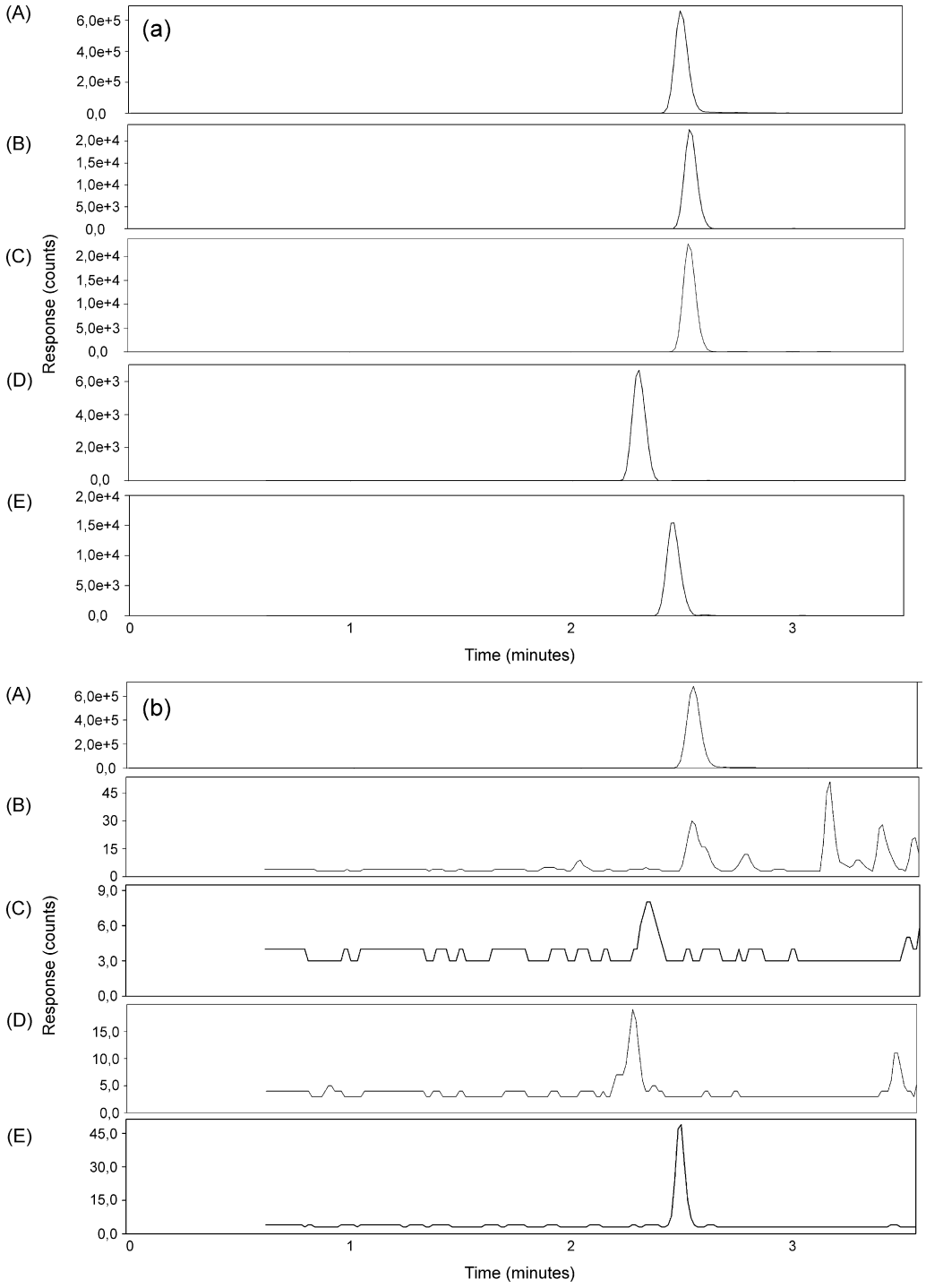

Fig. 2. (a) Chromatograms of a LLOQ sample of cyanoimipramine (A), clarithromycin (B), 14-hydroxyclarithromycin (C), 25-desacetylrifampicin (D), and rifampicin (E). (b) Chromatograms of a blank human plasma sample of cyanoimipramine (A), clarithromycin (B), 14-hydroxyclarithromycin (C), 25-desacetylrifampicin (D) and rifampicin (E).

Table 4
Results of accuracy, precision and dilution integrity [OTC: over-the-curve].

	Clarithromycin					14-Hydroxyclarithromycin					Rifampicin					25-Desacetylrifampicin				
	LLOQ	LOW	MED	HIGH	OTC	LLOQ	LOW	MED	HIGH	OTC	LLOQ	LOW	MED	HIGH	OTC	LLOQ	LOW	MED	HIGH	OTC
Concentration (mg/L)	0.1	0.2	5.0	8.0	25.0	0.1	0.2	5.0	8.0	25.0	0.2	1.0	2.5	4.5	30.0	0.2	1.0	2.5	4.5	30.0
Accuracy (%bias)	−5.4	0.2	−2.8	−0.4	0.0	−9.5	−0.3	1.5	3.3	5.0	−0.5	−3.5	−8.9	−9.8	−10.0	−6.3	−2.9	−4.5	−4.4	−6.0
Within-run precision (%CV)	7.7	3.9	2.5	3.0	2.0	5.3	6.3	2.5	2.5	3.1	5.8	2.5	4.4	2.8	4.7	9.3	5.7	5.2	4.0	7.2
Between-run precision (%CV)	4.5	2.9	0.3	0.0	0.8	6.7	0.0	0.9	3.1	0.0	0.0	1.9	3.5	1.1	3.1	5.4	0.0	2.5	3.3	6.4
Recovery (%)		111.4	100.1	105.9			116.8	101.3	108.3			109.1	109.4	101.3			116.9	106.0	104.1	

3.6. Recovery

The recoveries ranged from 100.1% to 116.9%, depending on the drug type and concentration (Table 4). The recovery of the internal standard is 94.7%.

3.7. Stability

The results of the stability experiments are summarized in Table 5.

The autosampler stability test showed that the processed samples can be re-injected after 3 days in the autosampler. The QC samples were not affected by three freeze–thaw cycles and storage in the refrigerator (5 °C) for 3 days. Storage of the rifampicin and 25-desacetylrifampicin QC samples at room temperature (on the desktop without light protection) for 1 day had little effect, but after 2 days the concentration already declined for about 25–30%. The clarithromycin and 14-hydroxyclarithromycin QC samples could be stored for 3 days at room temperature.

Table 6 shows the influence of light-protecting the rifampicin and 25-desacetylrifampicin QC samples stored at room temperature. The decline of the concentration of the QC samples exposed to light for 3 days was larger compared to the QC samples protected from light for 3 days. The QC samples exposed to light had a significant lower concentration than the QC samples protected from light (Mann–Whitney test, $p < 0.001$).

Clarithromycin and 14-hydroxyclarithromycin were not affected by either light exposure or light protection. Therefore the results of the clarithromycin and 14-hydroxyclarithromycin QC samples are not shown in Table 6.

3.8. Carryover

At concentrations above 40.0 mg/L of rifampicin and 25-desacetylrifampicin carryover was observed. Several flush and wash solutions (used in the injection device in the autosampler) were tried, but they did not have the desired effect. Therefore 5.0 mg/L was chosen as the upper value of the calibration curve. To minimize the carryover further, five blanks before the run and five blanks before the patient samples were placed in the run.

No carryover was observed for clarithromycin and 14-hydroxyclarithromycin at the maximum concentrations of the calibration curve.

3.9. Internal standards

The structure analogues of rifampicin and clarithromycin (roxithromycin and rifamycin SV) were intended to use as internal standards. However, the response of the components declined in time during validation tests and the chromatograms of rifamycin SV showed a degradation peak from which instability was concluded.

Therefore cyanoimipramine, a commonly used internal standard at the laboratory of the hospital pharmacy in the University Medical Center Groningen, was used. An autosampler stability test of 5 days showed an interday variability of 97–111% of the nominal response.

4. Discussion

This LC/MS/MS method for the determination of clarithromycin, 14-hydroxyclarithromycin, rifampicin and 25-desacetylrifampicin in human plasma is fully validated for selectivity, linearity, precision, accuracy, dilution integrity, matrix effects, recovery and stability. The QC samples are stable after three freeze–thaw cycles, 3 days in the autosampler and 3 days in the refrigerator. At room temperature clarithromycin and 14-hydroxyclarithromycin QC samples are stable for 3 days, while rifampicin and 25-desacetylrifampicin

Table 5
Results of stability experiments (%bias) [CLR, clarithromycin; 14-OH-CLR, 14-hydroxyclarithromycin; RIF, rifampicin; 25-DA-RIF, 25-desacetylrifampicin].

	CLR		14-OH-CLR		RIF		25-DA-RIF	
	LOW	HIGH	LOW	HIGH	LOW	HIGH	LOW	HIGH
Concentration (mg/L)	0.2	8.0	0.2	8.0	1.0	4.5	1.0	4.5
Autosampler (re-injection): 3 days	2.0	0.0	−0.4	3.0	−2.4	−12	−0.9	−4.0
Freeze–thaw: 3 cycles	1.3	−1.0	−2.7	2.0	−4.3	−6.0	−0.8	−1.0
Refrigerator: 3 days	0.9	−2.0	−3.0	2.0	−5.8	−7.0	−1.0	−2.0
Room temperature: 1 day	−2.8	−1.8	−3.0	0.6	−7.6	−9.1	−6.3	−9.8
Room temperature: 2 days	4.1	0.1	2.0	1.6	−26.9	−25.4	−29.8	−28.1
Room temperature: 3 days	10.7	−1.5	−4.9	−8.7	−38.3	−35.3	−41.9	−38.1

Table 6
Influence of light exposure on the concentration of rifampicin and 25-desacetylrifampicin at room temperature.

		Rifampicin		25-Desacetylrifampicin	
		LOW	HIGH	LOW	HIGH
Day 0	Mean concentration ± SD [range] (mg/L)	0.967 ± 0.014 [0.948–0.981]	4.534 ± 0.188 [4.34–4.83]	1.013 ± 0.052 [0.953–1.09]	4.948 ± 0.217 [4.74–5.28]
	Mean precision (%bias)	−2.4	1.7	3.2	12.1
Light exposed day 3	Mean concentration ± SD [range] (mg/L)	0.612 ± 0.025 [0.569–0.629]	2.884 ± 0.105 [2.77–3.00]	0.570 ± 0.019 [0.537–0.583]	2.730 ± 0.087 [5.59–2.82]
	Mean precision (%bias)	−38.3	−35.3	−41.9	−38.1
Light protected day 3	Mean concentration ± SD [range] (mg/L)	0.724 ± 0.028 [0.683–0.753]	3.312 ± 0.051 [3.25–3.39]	0.704 ± 0.044 [0.649–0.767]	3.228 ± 0.129 [3.09–3.43]
	Mean precision (%bias)	−26.9	−25.8	−28.2	−26.9

are stable for only 1 day. Plasma samples containing rifampicin and 25-desacetylrifampicin showed accelerated degradation in exposure to light. Instability of rifampicin and 25-desacetylrifampicin due to light and heat is described earlier [26,27] but the magnitude of the instability seems to be dependent of the local laboratory circumstances. Stability experiments have therefore to be performed in the local setting during method development. Samples should be protected from light and be analyzed directly or frozen at −20 °C.

Although, ranges of the calibration curves are sometimes wider [17,23], in our study these were based on the expected plasma concentrations of the components (depending on the dose and individual variation) and on the MIC of *Mycobacterium ulcerans*. Much lower LLOQs do not contribute significantly to the area under the concentration time curve and require liquid–liquid extraction (LLE) [17]. Due to the observed carryover of rifampicin the calibration range had to be narrowed compared to the method of analysis of Hartkoorn et al. [23]. However, the influence of carryover was minimized after scaling down the rifampicin and 25-desacetylrifampicin calibration curve to 0.20–5.0 mg/L and placing five blanks before the run and five blanks before the patient samples. Peak concentrations above 5.0 mg/L rifampicin and 25-desacetylrifampicin can be expected in the routine analysis. All patient samples should be diluted 10-fold with matrix before processing, which makes it possible to analyse all samples in one sequence. When the measured concentration of rifampicin and 25-desacetylrifampicin in the diluted sample is below 0.2 mg/L (similar to an undiluted concentration of 2.0 mg/L), the sample will be analyzed undiluted.

Cyanoimipramine is used to observe flaws in critical steps during sample preparation and chromatography. As in protein precipitation no classical extraction is performed, an structure analogue of the analyte is not mandatory to correct for extractions like LLE or solid phase extraction (SPE). The advantage of using a single agent as cyanoimipramine is that only one precipitation reagent is available in the laboratory and therefore mistakes are prevented. Moreover, cyanoimipramine has never been used as drug in patients. As long as the result of the method validation comply with the guidelines of FDA for method analysis this is acceptable.

5. Conclusion

A rapid and fully validated LC/MS/MS method was developed for the determination of clarithromycin, 14-hydroxyclarithromycin, rifampicin and 25-desacetylrifampicin in human plasma. The method is valued for 0.10–10.0 mg/L clarithromycin and 14-hydroxyclarithromycin and 0.20–30.0 mg/L rifampicin and 25-desacetylrifampicin. In case of high concentrations of rifampicin and 25-desacetylrifampicin (above 5.0 mg/L) the samples should be diluted 10-fold before processing due to a carryover risk.

This method is suitable for the evaluation of the expected pharmacokinetic drug interaction between clarithromycin and rifampicin.

Acknowledgements

The authors would like to thank Abbott (USA) and Sanofi-Aventis (Germany) for kindly providing the pure drug substances.

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