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Sensitive liquid chromatography–tandem mass spectrometry method for the determination of clarithromycin in human plasma

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Abstract

A sensitive method for the determination of clarithromycin in plasma is described, using high-performance liquid chromatographic separation with tandem mass spectrometric detection. Samples were prepared using liquid–liquid extraction and separated on a Supelco Discovery® C_{18} column with a mobile phase consisting of acetonitrile, methanol and acetic acid. Detection was performed by a PE SCIEX API 2000 mass spectrometer in the multiple reaction monitoring (MRM) mode (LC–MS–MS) using TurboIonSpray ionization and monitoring the transition of the protonated molecular ion for clarithromycin at m/z 748.5 (M+1) to the predominant product ion of m/z 158.2. The mean recovery of clarithromycin was 87.3%, with a lower limit of quantification of 2.95 ng/ml when using 0.3-ml plasma. This high-throughput method was used to quantify 230 samples per day, and is sufficiently sensitive to be employed in pharmacokinetic studies.

Keywords: Clarithromycin

1. Introduction

Clarithromycin (6-*O*-methylerythromycin) ($C_{38}H_{69}NO_{13}$) (Mr.=747.96) (Fig. 1) is a semi-synthetic 14-membered macrolide which displays a wide antibacterial spectrum. The effect of combining clarithromycin with a variety of other drugs for the treatment and prevention of disseminated *M. avium* infection in patients with AIDS is under investigation [7–10]. Abbott markets clarithromycin under the proprietary names ''Biaxin'', ''Klacid'' and ''Klaricid''. Its structure is identical to that of erythromycin, except that the *O*-methyl group has been substituted for a hydroxyl group at position six of the lactone [1]. Several methods for the determination of clarithromycin in plasma have been published [2–4], and a recent review article [5] lists many assay methods for the macrolide antibiotics in general. As is the case with many of the macrolide antibiotics, high-performance liquid chromatography (HPLC) with electrochemical detection was used [2–4] for the assay of clarithromycin because the molecule lacks a suitable chromophore which would make it amenable to UV detection. Although selective and highly sensitive, assay procedures making use of electrochemical detection is often very time consuming, both in the sample preparation steps and the chromatography. Thus, the sample to sample turn-around time in the published assay methods [2–4] for clarithromycin are of the order of 14, 12

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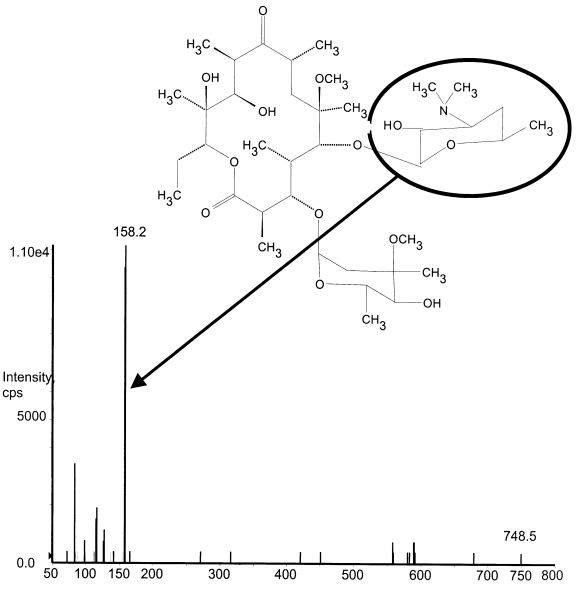

Fig. 1. A full scan spectrum (MS–MS) of a pure solution of clarithromycin in acetonitrile:0.1% formic acid (1:1, v/v). The parent [M+1] ion with m/z 748.5 and the predominant product ion m/z 158.2 is shown.

and 30 min, respectively, if one allows half a minute for the automated sample injection between each sample. Clearly, an assay procedure requiring less critical sample preparation procedures and a much faster turn-around time in the chromatographic process would be advantageous in clinical studies generating large numbers of samples. Although LC–MS–MS springs to mind immediately, no reference to any assay methods for clarithromycin using mass spectrometry as mode of detection could be found in the literature. This paper thus represents the first quantitative LC–MS–MS method for the determination of clarithromycin in human plasma using a relatively simple sample preparation procedure and a short turn-around time of only 3 min.

2. Experimental

2.1. Materials and chemicals

A Supelco Discovery® C_{18}, 5 μm, 150×2.1 mm, column (Supelco Park, Bellefonte, USA), heated at a constant temperature of 60 °C with a Shimadzu CTO-6A column oven (Shimadzu, Kyoto, Japan), was used for separation at a flow-rate of 0.26 ml/min (5 μl was injected onto the column). An Agilent 1100 quaternary pump delivered the mobile phase and the samples injected with an Agilent 1100 autosampler (Agilent, Palo Alto, CA). Detection was performed by a P-E Sciex API 2000 triple-quad mass spectrometer interfaced to an electrospray ionisation (ESI) TurboIonSpray source (Applied Biosystems Sciex, Ontario, Canada).

Acetic acid (Pro-Analysi) was obtained from Merck (Darmstadt, Germany); acetonitrile and methanol (BandJ High Purity) was obtained from Baxter (Muskegon, USA). All chemicals were used as received. Water was purified by RO 20SA reverse osmosis and Milli-Q® polishing system (Millipore, Bedford, MA, USA).

Clarithromycin (6-*O*-methylerythromycin) (Fig. 1) ($C_{38}H_{69}NO_{13}$) was supplied by ALPHARMA (Dalslandgade 11, Copenhagen, Denmark) and roxithromycin was obtained from the FARMOVS-PAREX-EL® Bioanalytical Services pure reference material library.

2.2. Preparation of calibration standards

Clarithromycin stock solutions (1579 μg/ml) were prepared in methanol and used immediately to spike blank plasma. Calibration standards and quality control standards were prepared in normal human plasma by spiking a pool of normal plasma to a known concentration and then serially diluting it with normal blank plasma to attain the desired concentration range (20 016–2.95 ng/ml). The prepared calibration standards and quality control standards were pipetted (500 μl) into polypropylene tubes and stored at −20 °C pending analysis.

2.3. Extraction procedure

To 300 μl plasma in a 10 ml glass ampoule was added 200 μl (0.1 *M*) $Na_2CO_3/NaHCO_3$ buffer solution (pH 9.2) and 100 μl roxithromycin internal standard solution (3.76 ng/ml in water). Hexane–ethyl acetate (1:1) (3 ml) was added, the samples vortexed for 60 s, and then centrifuged at 2500 *g* for 3 min at 10 °C. The aqueous phase was frozen on a Fryka Polar KP 250 cooling plate (Kältetechnik, Esslingen) at −30 °C for 3 min, the organic phase

decanted into a clean 5 ml glass ampoule and evaporated to dryness in a Savant SpeedVac® (Savant, Holbrook, NY, USA) rotary vacuum evaporator at ambient temperature. The extracts were reconstituted in 300 μl of acetonitrile–water (1:9), by vortexing for 30 s, transferred to autosampler vials, and 5 μl injected onto the HPLC column.

2.4. Liquid chromatography

Chromatography was performed at 60 °C, at a flow-rate of 0.26 ml/min with acetonitrile–methanol–acetic acid (0.1% in water) (25:25:50, v/v/v) as mobile phase. All chromatographic solvents were sparged with helium before use.

2.5. Mass spectrometry

Electrospray ionisation was performed in the positive mode with the nebulizing gas (nitrogen), curtain gas and turbospray set at 70, 50 and 70 l/h, respectively. The ionisation source was connected by a fused-silica (375 μm) capillary to the syringe pump for tuning, and the instrument response for clarithromycin was optimised using flow injection. Optimal response was obtained with a declustering potential setting of 61 V, a focusing potential of 230 V, entrance potential and exit potential of −8.0 and 8 V, respectively. The TurboIonSpray temperature was set at 400 °C.

The P-E Sciex API 2000 LC–MS–MS was operated at unit resolution in the multiple reaction monitoring (MRM) mode and the transitions of the protonated molecular ion for clarithromycin at m/z 748.5 to the predominant product ion m/z 158.2, and for roxithromycin m/z 837.6 to m/z 679.5 were monitored. The pause time was set at 5 ms and the dwell time at 150 ms. The relative collision energy was set at 39 eV. The mass spectrometer was interfaced to a computer workstation running Analyst™ software, version 1.0.

2.6. Validation

The method was validated by assaying clarithromycin plasma quality control samples in 6-fold at 3.12, 8.78, 23.3, 135, 359, 964, 2536, 6737 and 18 098 ng/ml to determine the accuracy and precision of the method. The quality control values were calculated from a standard regression curve composed of nine different concentrations spanning the concentration range 2.95–20 016 ng/ml for clarithromycin. Calibration graphs were constructed using peak area ratios of the relevant product ions versus nominal concentrations using Wagner ($\ln(y) = a(\ln(x)^2) + b(\ln(x)) + c$) ($r^2 = 0.9997$) (mean BIAS% = 0.1%) calibration curves.

2.7. Matrix effects

It has been noted that coeluting, undetected endogenous matrix components may reduce the ion intensity of the analyte and adversely affect the reproducibility and accuracy of the LC–MS–MS assay [6]. To determine whether this effect (called the matrix effect) is present or not, 10 different blank plasma pools were extracted and the reconstituted extracts then spiked with a known concentration of analyte. These samples were assayed and their peak areas compared. The reproducibility of the peak areas is an indication of the presence or absence of the matrix effect. The following data show that no significant matrix effect was observed in the samples assayed (RSD% = 2.36% for clarithromycin and 0.64% for roxithromycin) at the tested concentration of 1580 ng/ml for clarithromycin and 1315 ng/ml for roxithromycin.

2.8. Recovery

Absolute recovery of the analyte was determined in triplicate at high, medium and low concentrations in normal plasma by extracting drug free plasma samples spiked with clarithromycin. Recovery was calculated by comparison of the analyte peak-areas of the extracted samples with those of the unextracted analyte standards, representing 100% recovery.

3. Results and discussion

Initially protein precipitation was investigated as a possible sample preparation procedure. Then, 400 μl of acetonitrile was added to 200 μl of plasma in a microfuge tube. The samples were vortex mixed for

Table 1
Intra-day quality control results of clarithromycin

($n=6$)	QC I	QC H	QC G	QC F	QC E	QC D	QC C	QC B	QC A
Nominal (ng/ml)	18 098	6737	2536	964	359	135	23.3	8.78	3.12
Mean	16 393	6293	2513	992	369	139	23.4	8.61	3.33
% nom	90.6	93.4	99.1	102.9	102.6	102.6	100.6	98.1	106.8
C.V.%	4.0	5.4	5.7	4.8	3.4	3.3	4.5	9.8	4.3

30 s and centrifuged at 3000 *g* for 5 min at 10 °C. The supernatant aqueous layer (±200 μl) was transferred to an autosampler vial and 5 μl injected onto the HPLC column. An LLOQ of 8 ng/ml could be reached with this precipitation method.

The liquid–liquid extraction procedure described, however, was the method of choice. Although the LLOQ was found to be only 2.95 ng/ml (signal-to-noise (S/N) ratio = 27), this relatively high LLOQ was the result of carry-over (equivalent to an absolute amount of about 5 pg clarithromycin injected) inherent in the chromatographic instrument system which yielded a signal equivalent to an extract of an approximately 0.3 ng/ml sample. We have observed carry-over problems with different types of analytes on several occasions. Although it appears to be analyte-specific in many cases, the exact nature of the carry-over does not appear to be the same in all the cases that we have encountered. This phenomenon is the subject of continuous investigation in our laboratory. In the case of the clarithromycin, an LLOQ of about 0.4 ng/ml was reached when an extensive needle wash step with a stronger needle-wash solution was introduced to reduce carry-over (±2 ng/ml, S/N ratio = 16.0) substantially. However, this procedure would have increased the turn-around time for each analysis by an unacceptable margin while the LLOQ, which was attained (2.95 ng/ml), was totally acceptable considering the nature of the study and the range of concentrations studied (i.e. LLOQ to 1800 ng/ml).

Another observation was that the source of the P-E Sciex was ''saturating'' over a period of 12 h, which caused a drop in response down to a certain constant level of sensitivity. This was dealt with by injecting a series of 100 samples before the start of the analysis of the first study samples (getting the response to a constant level) and keeping on injecting samples in between batches (to keep the response at that level). This phenomenon is also presently the subject of intense investigations and will be reported on as soon as we have definitive results.

The mean absolute recoveries of clarithromycin, determined in triplicate at 8.78, 964 and 6737 ng/ml were 80.5, 90.8 and 90.5%, respectively. According to Kees et al. (1998) [11], the recovery of clarithromycin from water and plasma using liquid–liquid extraction was 80–90%.

Results presented in Table 1 indicate a valid calibration range of 2.95–20 016 ng/ml for clarithromycin. Table 2 depicts the quality control results obtained during the assaying of the study samples. On-instrument stability was inferred from intra-day quality control data obtained during the pre-study

Table 2
Inter-day quality control results of clarithromycin

	QC H	QC G	QC F	QC B	QC A
Nominal (ng/ml)	6829	2601	970	8.73	3.25
Mean	6677	2616	1023	8.61	3.07
C.V.%	5.7	2.5	4.6	6.8	0.8
n	6	6	6	5	6
% nom	97.8	100.6	105.4	98.7	94.6

validation. The ratio analyte/internal standard of 16 stability samples were compared during validation and inter-day validation, and no significant degradation could be detected in the cooled samples left on the autosampler for at least 21.1 h. To ascertain freeze–thaw stability, aliquots of quality control samples at 6829 ng/ml (QC G) and 8.73 ng/ml (QC B) were allowed to thaw and then immediately re-frozen. These quality controls were assayed the following day together with a set of the same quality controls (QC G and QC B) that had not been subjected to this additional freeze–thaw cycle. The results are summarised in Table 3. From these results we conclude that clarithromycin is stable in plasma over at least two freeze–thaw cycles.

Due to the high specificity of MS–MS detection, no interfering or late eluting peaks were found when assaying blank plasma extracts from six different sources when taking special precautions to avoid carry-over. As mentioned earlier, carry-over was a problem when time consuming special precautions was not taken.

Fig. 1 represents the single parent to product ion mass spectrum (MS–MS) of clarithromycin acquired with the most abundant product ion at m/z 158.2. A strong daughter ion with m/z 158.2 was also observed for roxithromycin, but to avoid cross talk, the transition to the even more abundant daughter ion with m/z 679.5 (Fig. 2) was optimised and monitored instead.

The P-E Sciex API 2000 LC–MS–MS detector was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ions m/z 748.5 and 837.6 to the principal product ions m/z 158 and 679.5 for clarithromycin and roxithromycin, respectively.

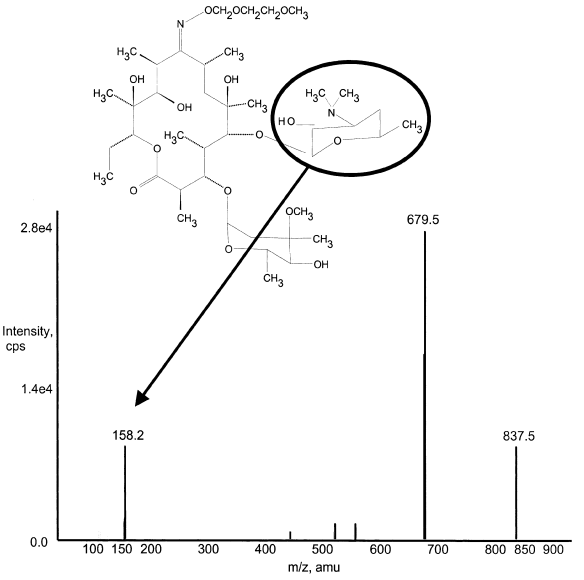

Fig. 2. A full scan spectrum (MS–MS) of a pure solution of roxithromycin (internal standard) in acetonitrile:0.1% formic acid (1:1, v/v). The parent [M+1] ions with m/z 837.5 and predominant product ion m/z 679.5 are shown.

Typical intra-batch retention times for clarithromycin were 1.87–1.92 min (mean RSD=0.30%) and for roxithromycin were 1.97–2.05 min (mean RSD=0.30%). A turn-around time of 3 min made it possible to analyse 230 samples per day. It took two analysts approximately 4–5 h to prepare the samples for analysis.

Table 3
Freeze–thaw stability, comparing a freeze–thaw–freeze–thaw (F/T/F/T) cycle to a freeze–thaw (F/T) cycle, using the ratio analyte/internal standard (ng/ml) at a high and a low concentration control

High (6737 ng/ml)	(F/T/F/T) (ng/ml)	(F/T) (ng/ml)	Low (8.73 ng/ml)	(F/T/F/T) (ng/ml)	(F/T) (ng/ml)
1	6678	6955	1	8.75	8.81
2	6893	6963	2	8.64	8.74
3	6773	6649	3	8.68	8.69
4	6842	7106	4	8.71	8.82
5	6801	7134	5	8.72	8.72
Mean	6797	6961		8.70	8.76
% Diff.	2.36			0.64	

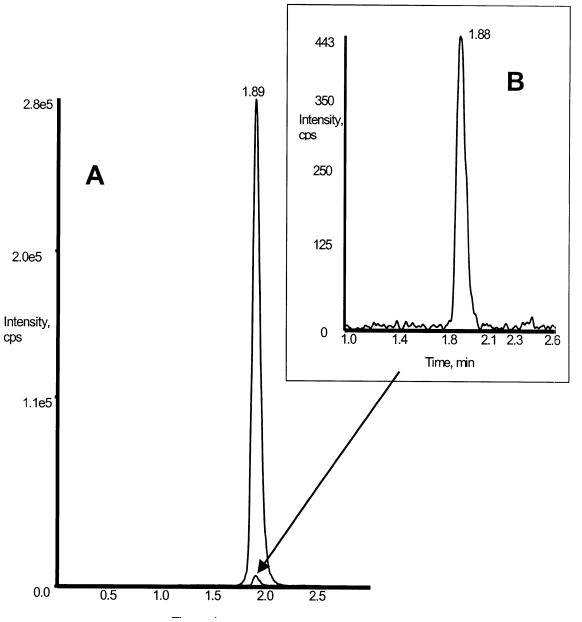

Fig. 3. Chromatograms of calibration standards, containing 20 016 ng/ml (A) and 2.95 ng/ml (B), respectively, of clarithromycin in plasma.

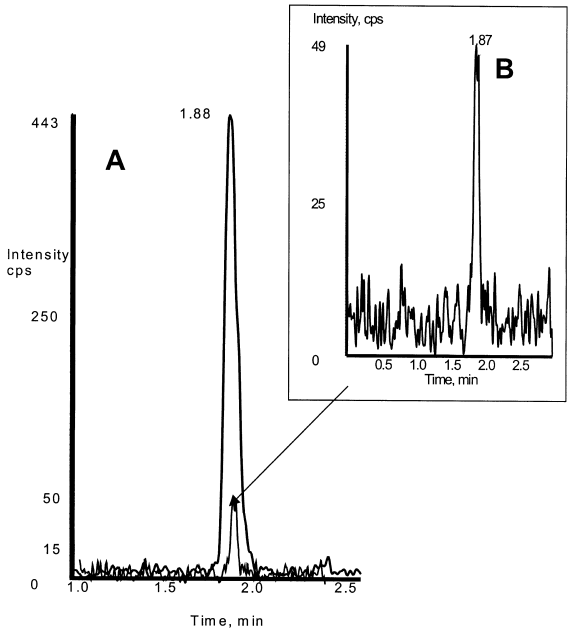

Fig. 4. Chromatograms of calibration standard containing 2.95 ng/ml of clarithromycin (A) in plasma and blank plasma extract (B), showing carry-over.

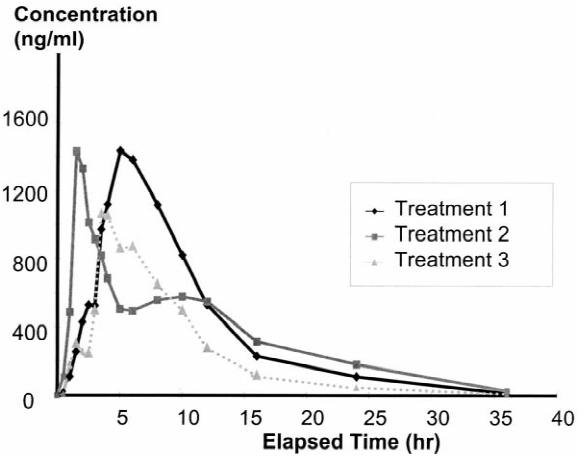

Fig. 5. Representative mean clarithromycin plasma concentration–time profile ($n=9$), as obtained after a single 500 mg oral dose of clarithromycin to human volunteers.

Fig. 3 shows representative chromatograms of clarithromycin obtained at 20 016 ng/ml and 2.95 ng/ml (LLOQ), while Fig. 4 depicts chromatograms from a calibration standard containing 2.95-ng/ml of clarithromycin, and a blank plasma extract (showing carry-over), magnified as an inset.

Fig. 5 represents the mean pharmacokinetic profiles obtained after a single dose of 500-mg clarithromycin of three different formulations of clarithromycin tested in nine healthy human volunteers. The maximum clarithromycin plasma concentrations obtained varied between 450 and 1800 ng/ml.

4. Conclusion

A highly selective method for the quantification of clarithromycin in human plasma has been developed and validated. Plasma concentrations of clarithromycin could be quantified from 2.95 to 20 016 ng/ml, making it possible to analyse samples up to 36 h after a single oral dose of 500 mg of clarithromycin to human volunteers. This is the first LC–MS–MS assay method described for the quantitation of clarithromycin in human plasma.

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