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Sensitive determination of erythromycin in human plasma by LC-MS/MS[1]

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Abstract

A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the analysis of erythromycin in human plasma (EDTA as anticoagulant) was developed and validated. The concentration ranges were 0.5–50 and 50–5000 ng ml^{-1}. The procedure involved alkalization of 0.5 ml of plasma, one step liquid-liquid extraction, dryness of the extract and reconstitution in 80:20 water:acetonitrile. An Inertsil ODS-2 5 μm, 3.0 × 50 mm column (Metachem) with a C_8 guard column and isocratic mobile phase were used for liquid chromatography. The mobile phase consisted of 1:1 acetonitrile:water with 2 mM NH_4OAc and 0.1% HOAc. A flow rate of 0.7 ml min^{-1} was used. The analysis time on LC-MS/MS for one sample was ≈2 min. A Turbo-Ionspray source was interfaced between the HPLC and triple quadrupole mass spectrometer (Sciex API III Plus). MS/MS analysis used Multi-Reaction Monitoring (MRM) mode. The lowest limit of quantitation (LOQ) was 0.5 ng ml^{-1} with all Quality Control (QC) sample recoveries varying between 88 and 105%. Nine intraday and interday calibration curves were generated yielding correlation coefficients ranging from 0.995 to 1.000. Average recovery for erythromycin at 1 ng ml^{-1} was 105% (±4.5%). Average recovery for the internal standard was 83–103%. Short-term and long-term stability in the freezer (−20°C), bench stability, and stability after 3 freeze/thaw cycles at −20 and −80°C were conducted. The samples were found to be stable under all conditions. The method developed and validated proved useful for clinical pharmacokinetic study sample analysis with high throughput due to its high sensitivity and very short analysis time.
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Keywords: Average recovery; Liquid chromatography-tandem mass spectrometry; Quality control

1. Introduction

Erythromycin is frequently used as an antibiotic drug. It is administered to human subjects as part of the clinical trial process, and the collected plasma samples are analyzed using a validated method. The derived plasma concentration data may be used as a part of an erythromycin phar-

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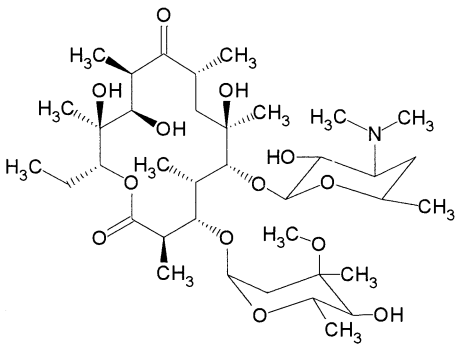
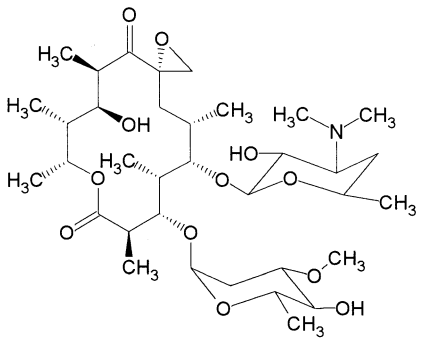


Erythromycin (MW 734)



Oleandomycin (MW 688)

Fig. 1. Structures of erythromycin ($M_W = 734$) and oleandomycin ($M_W = 688$).

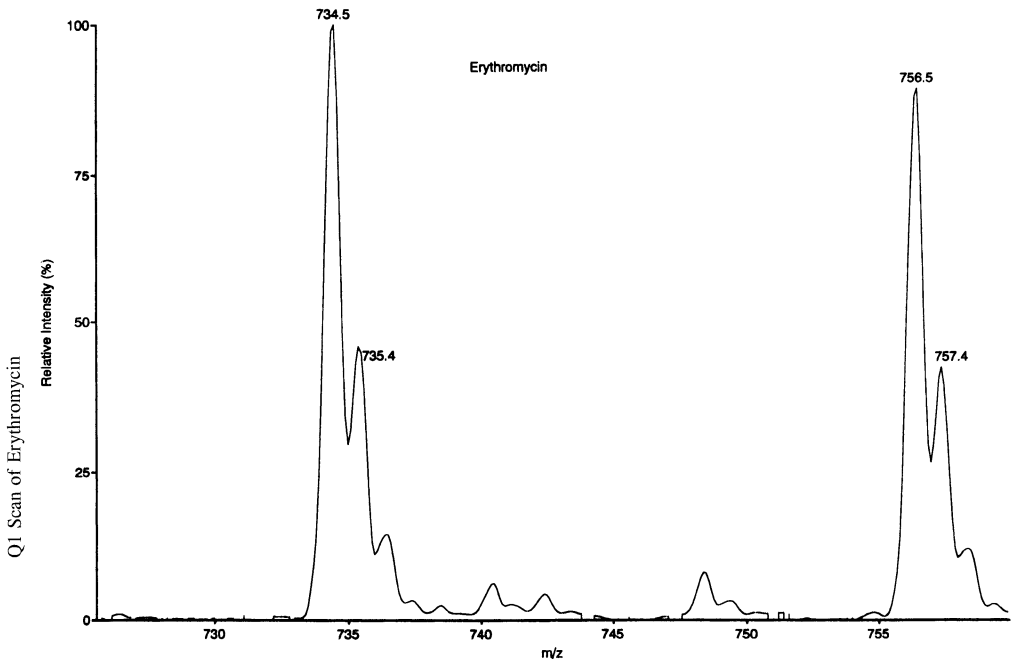


Fig. 2. Q1 scan of erythromycin.

macokinetic, bioequivalency, or drug interaction study.

HPLC techniques with UV, fluorescence, or electrochemical detection (ECD) were previously used for the detection of erythromycin concentration in human plasma [1–7]. The low limit of quantitation (LLOQ) was in the low ng ml^{-1} range [1]. The HPLC methods had runtimes that were too long (> 15 min) for analysis of large numbers of samples and also lacked specificity and ruggedness.

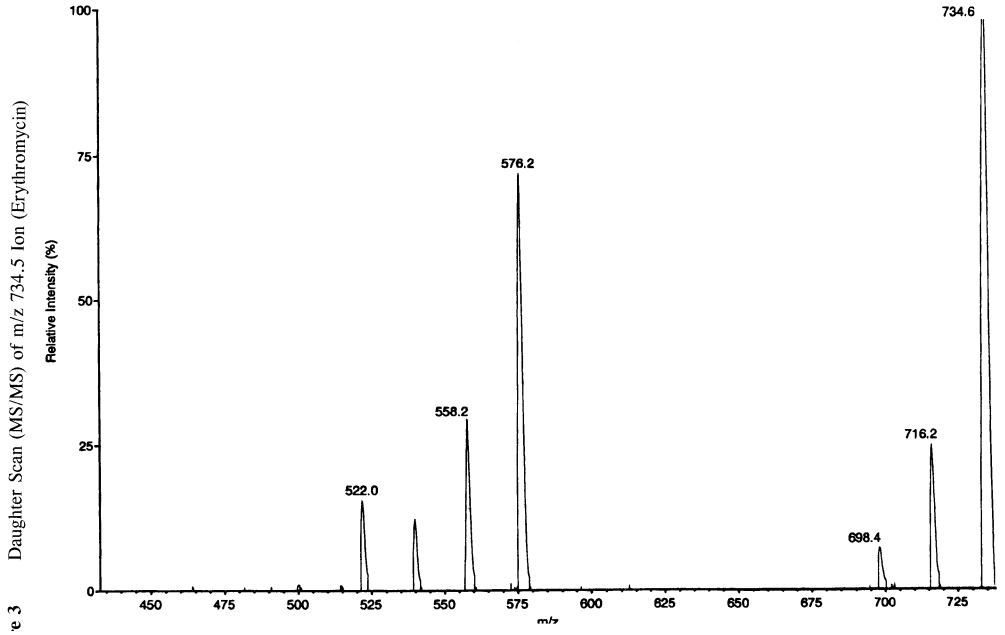


Fig. 3. Daughter scan (MS/MS) of m/z 734.5 ion (erythromycin).

The reported HPLC methods did not meet the needs of a clinical pharmacokinetic trial. An HPLC/MS method was developed by Lessard et al. [2], where the LLOQ was 20 ng ml^{-1}. A lower LLOQ and shorter runtime was still needed for analysis of clinical pharmacokinetic trial samples.

In light of the need for a rapid, more sensitive and specific assay for erythromycin in human plasma, a LC-MS/MS assay method has been developed and validated in this present study.

2. Experimental

2.1. Erythromycin

Erythromycin base (United States Pharmacopoeia, Rockville, MD) and the internal standard (IS), Oleandomycin, were fortified into human plasma with EDTA as anticoagulant. The molecular structure for erythromycin and oleandomycin is shown in Fig. 1.

2.2. Calibration curves

Split curves were used to cover a wide range of calibration. The concentrations of erythromycin were 0.5, 1.0, 2.5, 5.0, 10.0, 25.0 and 50.0 ng ml^{-1} for low curve and 50.0, 100, 250, 500, 1000, 2500 and 5000 ng ml^{-1} for high curve. The concentration of oleandomycin (IS) was 10 ng ml^{-1} for the low curve and 1000 ng ml^{-1} for the high curve. A plasma sample size of 0.5 ml was used for both curves.

2.3. Quality Control (QC) samples

For QC samples, 2.24, 20, and 40 ng ml^{-1} were used for low curve and 150, 2000, and 4000 ng ml^{-1} were used for high curve.

2.4. Extraction

All samples and fortified standard samples were vortexed 3–5 s before the extraction. Solvent A (50 μl) was added to each 0.5-ml plasma samples and standards. Solvent A was acetonitrile/methanol/0.05 M sodium acetate buffer (30/20/50;

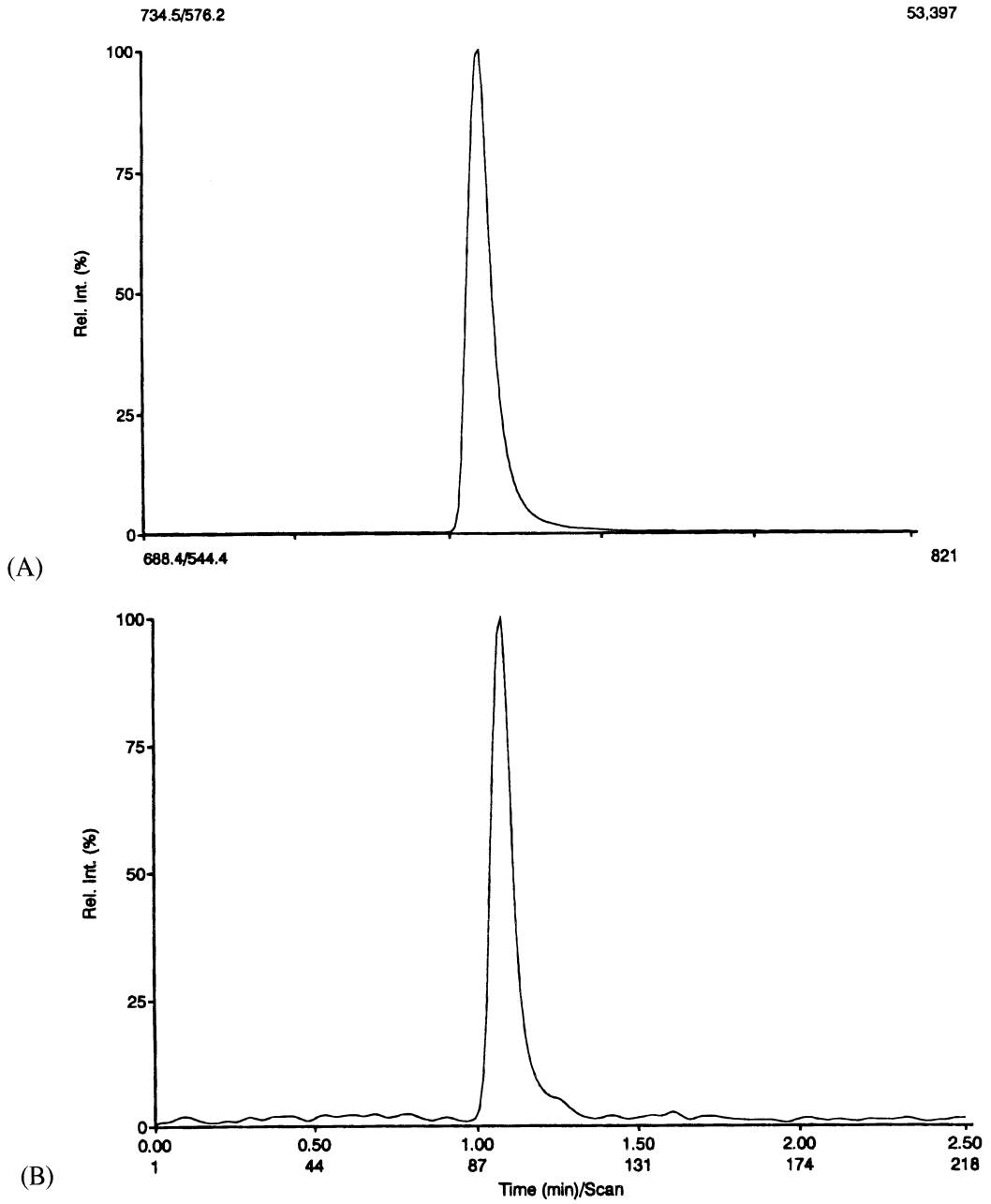


Fig. 4. A typical chromatogram of (A) erythromycin and (B) internal standard (50 ng ml^{-1}).

v/v/v) and the pH was adjusted to 6.30–6.40 with 1 N sodium hydroxide. After IS was added, the samples and standards were vortexed 3–5 s, while vortexing, 30 μl of saturated potassium carbonate was added with 3–5 s more of vortexing. *Tert*-butyl methyl ether (3 ml) was added to the

Table 1
Multiple intraday ($n = 5$) comparisons of the 10 ng ml^{-1} plasma calibration standard–LC-MS/MS calibration curve peak height, area, and retention times

	Erythromycin			Internal Standard		
	Height	Area	R.T. (min)	Height	Area	R.T. (min)
	20 395	140 432	0.57	1062	7424	0.54
	21 135	140 333	0.56	1056	6970	0.54
	18 927	124 432	0.56	882	5874	0.53
	22 979	151 504	0.55	1006	6342	0.53
	22 438	146 656	0.56	976	6249	0.54
Mean	21 175	140 671	0.56	996	6572	0.54
S.D.	1621	10 211	0.01	73	618	0.01
% CV	7.7	7.3	1.8	7.3	9.4	1.9

Intercept = 0.226
Slope = 1.548
Correlation Coefficient = 0.999

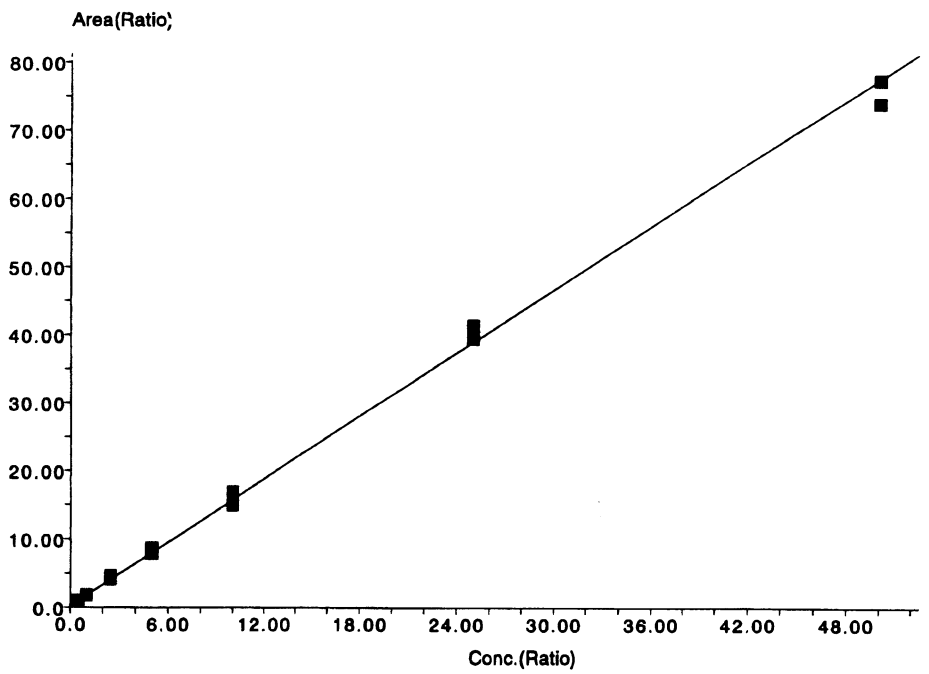


Fig. 5. A typical standard calibration curve (low range).

plasma, followed by vortexing in a multi-tube vortexer for 15 min. All samples and fortified standard samples were vortexed 3–5 s before the extraction. Extraction solvent (3 ml) were added and mixed in a Multi-Tube Vortexer for 15 min. After vortexing, the samples were centrifuged for 5 min at 2000 rpm in a Jouan C-412 Centrifuge. After centrifugation, the upper organic layer was transferred to a clean polypropylene tube. The sample extracts were dried under nitrogen at am-

Table 2
Back-calculated concentrations of high range standards (50–5000 ng ml^{-1})

Theoretical concentration (ng ml^{-1})	Back-calculated concentration (ng ml^{-1})						
Validation day	50	100	250	500	1000	2500	5000
Day 1	40.4	111.5	263.7	522.2	1044.1	2439.1	4598.4
	46.4	95.0	284.4	550.3	1003.5	2468.4	5355.8
	41.8	100.9	247.7	503.6	1020.3	2514.5	4947.9
Mean	42.9	102.5	265.3	525.4	1022.6	2474.0	4967.4
S.D.	3.1	8.4	18.4	23.5	20.4	38.0	379.1
% CV	7.2	8.2	6.9	4.5	2.0	1.5	7.6
% R.E.	−14.2	2.5	6.1	5.1	2.3	−1.0	−0.7
Day 2	54.2	99.1	247.9	523.3	982.3	2550.8	4972.4
	54.1	97.6	232.4	487.9	1000.0	2567.4	5053.9
	40.9	102.4	269.8	501.6	1011.0	2420.8	4930.4
Mean	49.7	99.7	250.0	504.3	997.8	2513.0	4985.6
S.D.	7.7	2.5	18.8	17.9	14.5	80.3	62.8
% CV	15.5	2.5	7.5	3.5	1.5	3.2	1.3
% R.E.	−0.6	−0.3	0.0	0.9	−0.2	0.5	−0.3
Day 3	57.4	98.9	240.9	519.5	994.6	2624.1	5033.0
	42.2	103.9	254.5	536.2	1069.1	2728.5	4827.7
	44.2	94.1	237.4	503.8	1019.0	2372.0	4799.1
Mean	47.9	99.0	244.3	519.8	1027.6	2574.9	4886.6
S.D.	8.3	4.9	9.0	16.2	38.0	183.3	127.6
% CV	17.3	4.9	3.7	3.1	3.7	7.1	2.6
% R.E.	−4.2	−1.0	−2.3	4.0	2.8	3.0	−2.3
Grand mean	46.8	100.4	253.2	516.5	1016.0	2520.6	4946.5
S.D.	6.6	5.2	16.8	19.3	26.6	111.0	207.5
% CV ($n = 9$)	14.1	5.2	6.6	3.7	2.6	4.4	4.2
% R.E.	−6.4	0.4	1.3	3.3	1.6	0.8	−1.1

bient temperature, and the dried extracts were reconstituted in 80:20 water:acetonitrile (1 ml for samples in the low curve concentration range and 40 ml for samples in the high curve concentration range) and vortexed for 10–15 s.

2.5. Chromatography

The reconstituted extracts (20 μl) were injected onto a 3.0×50-mm Inertsil ODS-2 column (Metachem) with a C_8 guard column. Mobile phase was 50:50 water:acetonitrile with 2 mM ammonium acetate and 0.1% acetic acid. The HPLC was operated in isocratic mode with a flow rate of 0.7 ml min^{-1}. The retention times for erythromycin and oleandymycin were ≈ 1.05 and 1.08 min, respectively, with a total runtime of 2 min. The analyte erythromycin and oleandomycin were eluted into the Turbo IonSpray source of a PE Sciex API III Plus triple quadrupole mass spectrometer.

2.6. Mass spectrometry conditions

Instrument: PE API III Plus triple quadrupole mass spectrometer with the Turbo IonSpray source.
Ion Detection: Positive for both erythromycin and oleandomycin.
Mode: Multiple Reaction Monitoring (MRM).
Turbo Probe Temperature: 500°C.
Turbo Auxiliary Gas Flow (N_2): 6 L min^{-1}.
Orifice Potential: 60 V.
Collision gas: 10% nitrogen + 90% Argon mixture (set at 2.75×10^{15} atoms/cm^2)
Collision energy: 12 V

Erythromycin and oleandomycin were detected by monitoring specific precursor-product ion tran-

Table 3
Back-calculated concentrations of low range standards (0.5–50 ng ml^{-1})

Theoretical concentration (ng ml^{-1})	Back-calculated concentration (ng ml^{-1})						
Validation Day	0.50	1.00	2.50	5.00	10.00	25.00	50.00
Day 1	0.47	1.01	2.35	4.46	11.37	24.88	48.69
	0.48	1.00	2.32	4.96	11.34	25.06	46.64
	0.39	1.05	2.75	5.24	11.29	26.28	49.95
Mean	0.45	1.02	2.47	4.89	11.33	25.41	48.43
S.D.	0.05	0.03	0.24	0.40	0.04	0.76	1.67
% CV	11.1	2.9	9.7	8.2	0.4	3.0	3.4
% R.E.	−10.0	2.0	−1.2	−2.2	13.3	1.6	−3.1
Day 2	0.54	0.91	2.42	4.72	11.82	26.66	46.88
	0.42	0.92	2.53	5.34	11.90	23.64	48.31
	0.40	0.89	2.46	5.43	11.80	26.53	47.46
Mean	0.45	0.91	2.47	5.16	11.84	25.61	47.55
S.D.	0.08	0.02	0.06	0.39	0.05	1.71	0.72
% CV	17.8	2.2	2.4	7.6	0.4	6.7	1.5
% R.E.	−10.0	−9.0	−1.2	3.2	18.4	2.4	−4.9
Day 3	0.40	0.86	2.42	4.75	10.89	22.41	46.00
	0.37	1.02	2.59	4.94	11.60	24.11	46.97
	0.57	1.03	2.57	5.34	12.21	27.58	53.37
Mean	0.45	0.97	2.53	5.01	11.57	24.70	48.78
S.D.	0.11	0.10	0.09	0.30	0.66	2.64	4.00
% CV	24.4	10.3	3.6	6.0	5.7	10.7	8.2
% R.E.	−10.0	−3.0	1.2	0.2	15.7	−1.2	−2.4
Grand mean	0.45	0.97	2.49	5.02	11.58	25.24	48.25
S.D.	0.07	0.07	0.13	0.34	0.40	1.67	2.27
% CV ($n=9$)	15.6	7.2	5.2	6.8	3.5	6.6	4.7
% R.E.	−10.0	−3.0	−0.4	0.4	15.8	1.0	−3.5

sitions. For erythromycin, the ion transition (parent → daughter pair) was m/z 734.5 → 576.2. For oleandomycin, the ion transition was 688.4 → 544.4.

3. Results and discussion

3.1. Mass spectrometry

The mass spectrometry data were first collected in Q1 scan for erythromycin by using flow injection analysis. The resulting spectrum was dominated by ions attributable to erythromycin $[M+1]^+$, m/z 734.5 and $[M+Na]^+$, m/z 756.6 (Fig. 2). The production spectrum of m/z 734.5 was then further collected in the daughter (MS/MS) scan. A significant product ion was observed at m/z 576.2 (Fig. 3) which was the fragmentation ion from having one single sugar moiety lost from the parent ion m/z 734.5. Therefore, parent ion m/z 734.5 (as a precursor ion) and a characteristic daughter ion m/z 576.2 (as a product ion) were chosen as the precursor → product transition pair for MRM mode detection to quantify the erythromycin concentration. A typical ion chromatogram is shown in Fig. 4. The same process was conducted on internal standard oleandomycin to determine the ion pair 688.4 → 544.4.

3.2. Calibration curve

The method was shown to be very reproducible within a run in terms of the peak shape, height, area, and retention time, with % CVs ranging from 0.5 to 9.4% (Table 1). Over a concentration range of 0.5–50 and 50–5000 ng ml^{-1}, the peak area ratio of analyte to oleandomycin was fitted

to a weighted linear equation (weighted 1/concentration). The correlation coefficients (r) obtained for all separate calibration curves ($n = 9$) were 0.995 or greater. A typical calibration curve is shown in Fig. 5.

Back-calculated concentrations and statistics for calibration standards, shown in Tables 2 and 3, indicated that curve fit was appropriate, and the method was accurate (%R.E. $< 15\%$; $< 20\%$ for LOQ) and precise (%CV $< 15\%$; $< 20\%$ for LOQ) within-day and over the course of the 3-day validation.

Intraday accuracy (%R.E.) and precision (%CV) for all 3 days show appropriate accuracy and precision in their back-calculated values ($n = 9$). Therefore, the LLOQ in present method is 0.5 ng ml^{-1} for erythromycin [2].

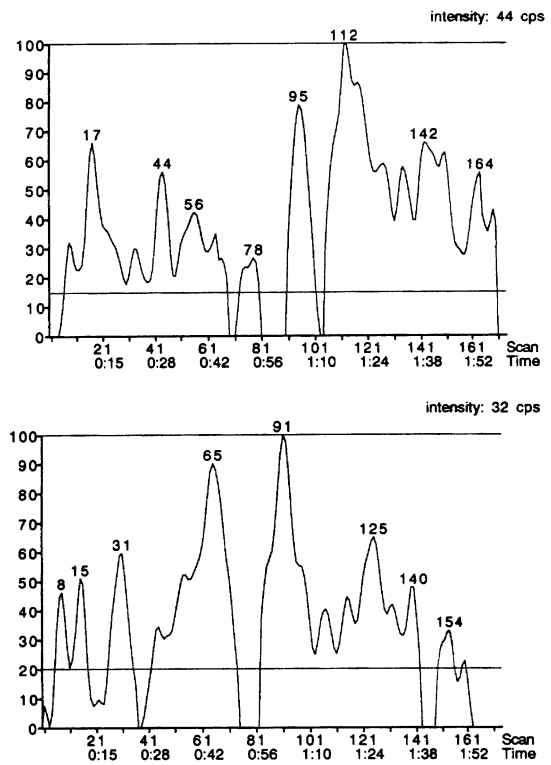


Fig. 6. Plasma blank chromatogram (no interference peak).

Table 4
Measured concentrations of high range QC samples

Theoretical concentration (ng ml^{-1})	Measured concentration (ng ml^{-1})		
Validation day	150	2000	4000
Day 1	173.7	1964.7	4166.3
	161.8	1998.8	4380.9
	138.6	2049.6	4283.6
	154.1	2088.6	4363.6
	154.5	2276.8	4337.1
	142.9	2159.0	4174.4
Mean	154.3	2089.6	4284.3
S.D.	12.7	114.3	94.2
% CV ($n = 6$)	8.2	5.5	2.2
% R.E.	2.9	4.5	7.1
Day 2	158.7	1872.9	3495.7
	151.0	1718.4	3600.9
	146.5	1798.0	3502.2
	140.0	1873.7	3866.4
	143.8	1833.6	3514.8
	134.7	1905.6	3601.1
Mean	145.8	1833.7	3596.9
S.D.	8.4	67.6	140.4
% CV ($n = 6$)	5.8	3.7	3.9
% R.E.	−2.8	−8.3	−10.1
Day 3	150.4	2152.1	3867.0
	136.6	1839.8	3817.5
	148.1	2013.5	3860.6
	147.6	1924.0	3774.4
	145.3	1939.8	3377.7
	134.9	1932.2	3611.1
Mean	143.8	1966.9	3718.1
S.D.	6.5	106.2	191.2
% CV ($n = 6$)	4.5	5.4	5.1
% R.E.	−4.1	−1.7	−7.0
Grand mean	148.0	1963.4	3866.4
S.D.	10.1	141.7	338.0
% CV ($n = 18$)	6.8	7.2	8.7
% R.E.	−1.3	−1.8	−3.3

3.3. Selectivity/specificity

Plasma from six different sources, and pooled plasma from multiple sources were found to be free from interfering parent and product ions at the retention times of erythromycin and oleandomycin. In addition, no interfering ions resulted from either oleandomycin or a tested co-administered drug, nor did any interfering ions result from erythromycin or the co-administered drug

that would interfere with the oleandomycin analysis. Representative chromatograms are presented in Fig. 6.

3.4. QC samples and recovery

Between-run precision and accuracy were assessed by analyzing a total of six quality control (QC) samples in six replicates on three separate days. Measured concentrations for the high and low range QC samples and the statistics, shown in Tables 4 and 5, respectively, demonstrate that the method was precise and accurate for both intraday and interday. Representative chromatograms for the low, mid, and high QC samples for the high range of calibration curve are presented in Figs. 7–9, respectively.

Table 5
Measured concentrations of low range QC samples

Theoretical concentration (ng/mL)	Measured concentration (ng/mL)		
Validation day	2.24	20.0	40.0
Day 1	1.92	21.3	40.4
	1.78	20.5	43.9
	1.83	21.1	40.3
	1.81	21.0	39.2
	1.69	20.6	41.9
	1.69	19.6	39.2
Mean	1.79	20.7	40.8
S.D.	0.09	0.6	1.8
% CV ($n=6$)	0.05	2.9	4.4
% R.E.	−20.0	3.5	2.0
Day 2	2.00	22.9	43.8
	1.94	22.1	41.4
	1.89	20.2	38.4
	2.05	20.1	42.0
	1.92	20.7	40.5
	1.94	23.9	43.9
Mean	1.96	21.7	41.7
S.D.	0.06	1.6	2.1
% CV ($n=6$)	3.1	7.4	5.0
% R.E.	−12.5	8.5	4.3
Day 3	2.81	21.3	39.2
	2.80	22.2	40.7
	2.64	14.5	37.5
	2.80	21.3	41.0
	2.63	19.5	40.0
	2.47	22.0	41.0
Mean	2.69	20.1	39.9
S.D.	0.14	2.9	1.4
% CV ($n=6$)	5.2	14.4	3.5
% R.E.	20.0	0.5	−0.3
Grand mean	2.15	20.8	40.8
S.D.	0.41	1.9	1.8
% CV ($n=18$)	19.3	9.1	4.4
% R.E.	−4.0	4.0	2.0

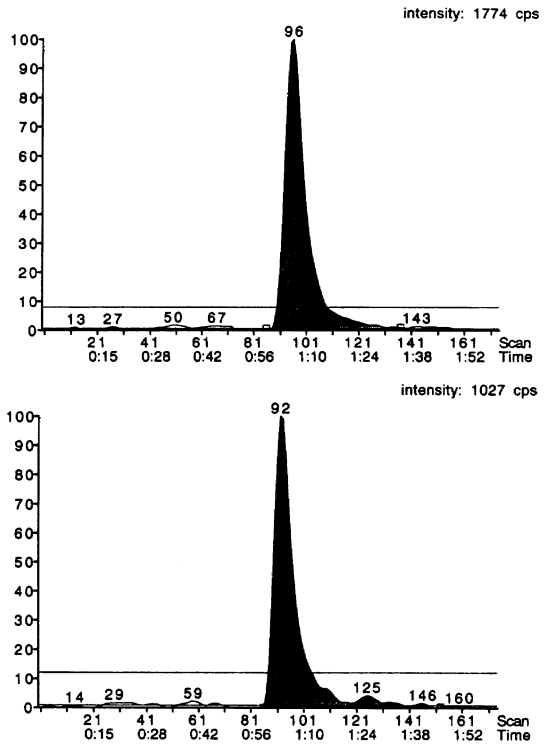


Fig. 7. Low QC sample chromatogram for the high range of calibration curve (150 ng ml^{-1}).

Extraction recoveries were performed by comparing fortified reconstituted post-extract (equal to 100% extraction recovery) and processed plasma sample peak areas for erythromycin or oleandomycin. The recoveries at 1 ng ml^{-1} were an average of 105% with CV = 4.5% ($n=3$). Average recovery for the oleandomycin ranged from 83 to 110%.

3.5. Stability

Back-calculated concentrations for stability samples were compared to freshly prepared QC samples (Time Zero Samples). Data for 1, 4, and

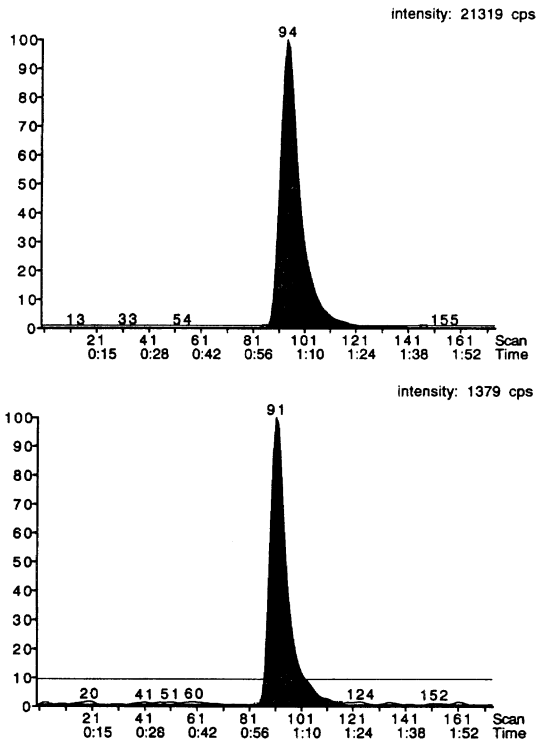


Fig. 8. Mid QC sample chromatogram for the high range of calibration curve (2000 ng ml − 1).

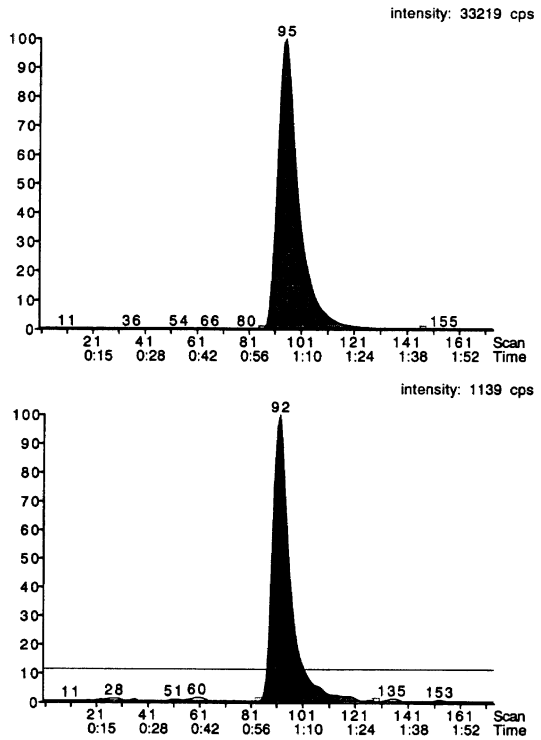


Fig. 9. High QC sample chromatogram for the high range of calibration curve (4000 ng ml^{-1}).

20 h at room temperature and 3 freeze/thaw cycles at ≈ -20 and -80°C, as well as short-term and long-term stability indicate that erythromycin is stable under all conditions studied.

4. Conclusion

In conclusion, this LC-MS/MS method for the analysis of erythromycin in human plasma was selective, sensitive, accurate, precise, and linear over the two concentration ranges studied (0.5–50 and 50–5000 ng ml^{-1}). Average recovery was at least 80%, and LLOQ was 0.5 ng ml^{-1}. Samples were shown to be stable in conditions under which clinical samples would be stored and processed. Runtime of one sample was only 2 min which was very beneficial to clinical and pharmacokinetic studies.

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