

Simultaneous determination of erythromycin propionate and base in human plasma by high-performance liquid chromatography–electrospray mass spectrometry

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Abstract

An analytical method for simultaneous determination of erythromycin propionate and its active metabolite, erythromycin base, in human plasma by high-performance liquid chromatography–electrospray mass spectrometry (HPLC–ESI–MS) was developed and validated. Roxithromycin was selected as the internal standard. The samples were directly injected after simple deproteinized procedure only. The separation was achieved on a Johnson Spherigel analytical column packed with 5 μm C₁₈ silica, employing acetonitrile–0.1% formic acid aqueous solution (50:50) as mobile phase. The quantification of target compounds was obtained by using a selected ion monitoring (SIM) at m/z 790.7 for erythromycin propionate, m/z 734.7 for erythromycin base and m/z 837.8 for roxithromycin. The correlation coefficients of the calibration curves were better than 0.997 ($n=6$), in the ranges from 2 ng/ml to 1 $\mu\text{g/ml}$, and from 1 to 10 $\mu\text{g/ml}$ for erythromycin propionate and base. The method can provide the necessary sensitivity, precision and accuracy to allow the simultaneous determination of both compounds in a patient's plasma following a single administration of erythromycin stinoprate capsule (500 mg erythromycin base equivalent).

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1. Introduction

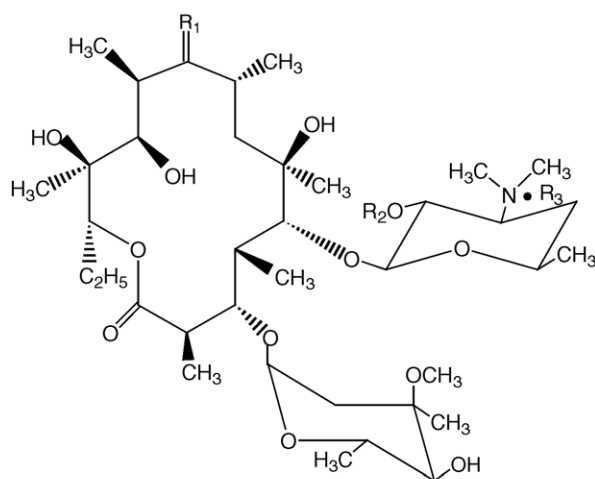
Erythromycin is a macrolide antibiotic, which is used mainly in the treatment of infections caused by Gram-positive and some Gram-negative organisms [1]. Erythromycin base is rapidly degraded in acidic media such as in gastric juice [1–4]. To avoid decreasing gastric inactivation of the drug, it is necessary to use structurally modified erythromycin derivatives or acid-resistant dosage forms.

Erythromycin stinoprate (propionate-*N*-acetylcysteinate) (Fig. 1) is the acetylcysteinate salt of propionyl ester of ery-

thromycin which is reported to be acid-stable due to its insolubility in acidic media. It is believed that erythromycin stinoprate is more effective than erythromycin [5,6]. In the less acidic environment of the small intestine, the drug dissociates to the soluble propionyl ester. The drug is primarily absorbed as the intact ester, and a less amount being absorbed as erythromycin base formed by the hydrolysis of the ester in the small intestine. To obtain the necessary antibacterial activity, the propionyl ester, which is the major part of the circulating drug, must be hydrolyzed *in vivo* into erythromycin base.

To study the bioavailability and pharmacokinetics of erythromycin stinoprate, the simultaneous determination of erythromycin propionate and base in plasma is very important.

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	R ₁	R ₂	R ₃
Roxithromycin	NOCH ₂ OCH ₂ CH ₂ OCH ₃	H	
Erythromycin base	O	H	
Erythromycin propionate	O	CH ₃ CH ₂ CO	
Erythromycin stinoprate	O	CH ₃ CH ₂ CO	CH ₃ CONHCH(CH ₂ SH)COOH

Fig. 1. Chemical structures of erythromycin stinoprate, its propionyl ester, the free base and roxithromycin.

To our knowledge, some methods such as bioautography [7,8], fluorimetry [9] coupled with a microbiological endpoint [10–12], microbiological and high-performance liquid chromatography (HPLC) analytic methods [6] were developed. But these methods all suffered from the general disadvantages of the microbiological methods [13,14] as well as low sample throughputs. A HPLC analytical method coupled with coulometric detection [4] was also developed. But in this method solid phase extraction (SPE) was involved and the lower limit of quantification (LLOQ) was only 250 ng/ml.

More recently, LC-API-MS has replaced GC/MS to study drug metabolism [15]. In this paper, a HPLC-electrospray ionisation-mass spectrometry (ESI-MS) method for simultaneous analysis of erythromycin propionate and base in human plasma was developed. The method has the necessary sensitivity, precision and accuracy to allow the simultaneous determination of both compounds in a patient's plasma following a single administration of erythromycin stinoprate capsule (500 mg erythromycin base equivalent). This method is also much simpler, reagent-saving and time-saving than the reported methods [6–12]. Its specifications are suitable for assessing bioavailability and pharmacokinetics of the erythromycin stinoprate.

2. Experimental

2.1. Instrumentation and experimental conditions

A Waters (Milford, MA, USA) Alliance 2695 liquid chromatographic system interfaced to a Micromass ZQ 2000 ESI mass spectrometer and a Masslynx™ 3.5 data system was equipped with a Johnson Spherigel analytical column (250 mm × 4.6 mm) (Dalian, China) packed with 5 μm C₁₈ silica. Acetonitrile – 0.1% formic acid aqueous solution (50:50) were used as the mobile phase. The column temperature was maintained at 30 °C. The flow rate was 0.8 ml/min. The outlet of the UV detector was split, and only 0.2 ml/min portion of the column eluent was delivered into MS. The mass spectrometer was operated in the positive mode to generate [M + H]⁺ ions at *m/z* 790.7 for erythromycin propionate, *m/z* 734.7 for erythromycin base and *m/z* 837.8 for roxithromycin. Nitrogen was used as desolvation gas at a flow rate of 200 l/h and cone gas at a flow rate of 50 l/h. The desolvation temperature was 200 °C. Capillary and cone voltages were 4000 and 40 V, respectively. The ionization source was worked at 105 °C. Extractor voltage was set at 5 and 1 V for RF lens. An Eppendorf 5804R Multipurpose Centrifuge (Brinkmann, Westbury,

NY) worked at $27,000 \times g$ to remove proteins from samples.

2.2. Chemicals and reagents

Erythromycin propionate, erythromycin base and internal standard roxithromycin were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The purity of the standards was higher than 99%. Structures of the compounds are shown in Fig. 1.

HPLC-grade acetonitrile purchased from Tedia company, Inc. (Fairfield, Ohio, USA), and ultrapure water prepared by a Millipore Milli-Q purification system (Millipore Corp. Bedford, MA) were used as the mobile phase of HPLC/MS. Other reagents were of analytical-grade. Drug-free and drug-containing plasma was obtained from volunteers and patients, respectively. Plasma was stored below -70°C until used for analysis.

2.3. Stock solutions

The stock solution of erythromycin propionate was prepared by dissolving 10.3 mg in 10 ml of ethanol. The stock solution of erythromycin base was prepared by dissolving 10.2 mg in 10 ml of ethanol. The stock solution of internal standard roxithromycin was prepared by dissolving 30 mg in 10 ml of acetonitrile. A series of stock standard solutions, which were used for preparing calibration standards and quality control (QC) samples, were then gained by the appropriate dilutions of the above-mentioned stock solutions with ethanol to reach concentrations of 10, 100, 500 $\mu\text{g/ml}$ for erythromycin propionate and base. Further dilution of the stock solution of roxithromycin with acetonitrile resulted in the spiked internal standard solution (0.3 $\mu\text{g/ml}$). All the stock solutions were stored in the dark under refrigeration at -70°C .

2.4. Quality control samples

Quality control samples were prepared at three different concentration levels, the lowest (2 ng/ml), near the middle (5 $\mu\text{g/ml}$) and the highest (10 $\mu\text{g/ml}$). QC samples were prepared daily by spiking each proper volume stock solutions to 0.5 ml drug-free plasma to obtain the last concentrations equivalent to 2 ng/ml, 5 and 10 $\mu\text{g/ml}$ of erythromycin base and propionate. And then after being added 1 ml of 0.3 $\mu\text{g/ml}$ roxithromycin internal standard stock solution and being vortex mixed for 3–5 s, all the samples were centrifuged at $27,000 \times g$ for 10 minutes at 4°C . A 10 μl aliquot of the supernatant of each sample was then directly injected on the Spherigel analytical column.

2.5. Calibration standard solutions

Proper volume of stock standard solutions of erythromycin propionate and base was added to 0.5 ml of drug-free human

plasma to obtain calibration standards concentrations ranging from 2 ng/ml to 10 $\mu\text{g/ml}$ for erythromycin propionate and base in polypropylene tubes. Daily calibration standards of erythromycin propionate and base were prepared at least 12 different concentrations. And then after being added 1 ml of 0.3 $\mu\text{g/ml}$ roxithromycin internal standard stock solution and being vortex mixed for 3–5 s, all the samples were centrifuged at $27,000 \times g$ for 10 minutes at 4°C . A 10 μl aliquot of the supernatant of each sample was then directly injected on the Spherigel analytical column. Every calibration standard was injected three times.

2.6. Sample preparation

0.5 ml plasma samples obtained from patients after the administration of a single erythromycin stinoprate capsule (500 mg base equivalent) together with 1 ml of 0.3 $\mu\text{g/ml}$ roxithromycin internal standard stock solution were added to polypropylene tubes. After vortex mixed for 3–5 s, all the samples were then centrifuged at $27,000 \times g$ for 10 min at 4°C . A 10 μl aliquot of the supernatant of each sample was then directly injected on the Spherigel analytical column.

3. Results and discussion

3.1. The choosing of internal standard

More recently, LC-API-MS has replaced GC/MS to study drug metabolism [15]. One of the limitations, however, is the susceptibility of API interfaces to co-eluting residual matrix components [16,17]. Matrix effect, which will possibly lead to ion suppression, often adversely affects accuracy and precision of the method. One of the solutions to the problem is to look for a suitable internal standard, which has very similar characteristic, and identical, or at least very close, retention time [18,19]. Roxithromycin, whose structure is similar to target compounds, was proved to be favorable in obtaining acceptable accuracy and precision of the method.

3.2. Specificity

The specificity of the analytical method was checked by preparing and analyzing six different batches of blank plasma samples. The specificity was assessed by comparing the apparent signals for erythromycin base and propionate in six blank plasma samples, which were spiked with standard solutions of different concentrations in the range of 2 ng/ml to 10 $\mu\text{g/ml}$ added, to the response of blank plasma samples. The blank plasma sample showed a clean baseline at m/z 790.7, 734.7 and 837.8 at the relevant retention time and no endogenous interferences were observed. Typical selected ion monitoring (SIM) current profiles of the results were shown in Figs. 2 and 3. These profiles showed that the method adopted was applicable.

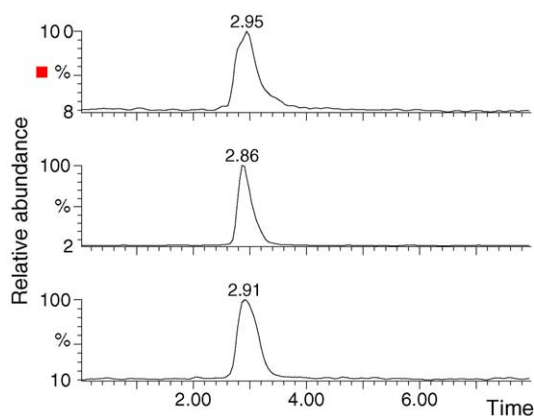


Fig. 2. SIM current profiles at m/z 790.7, 837.8 and 734.7, respectively, of blank human plasma sample.

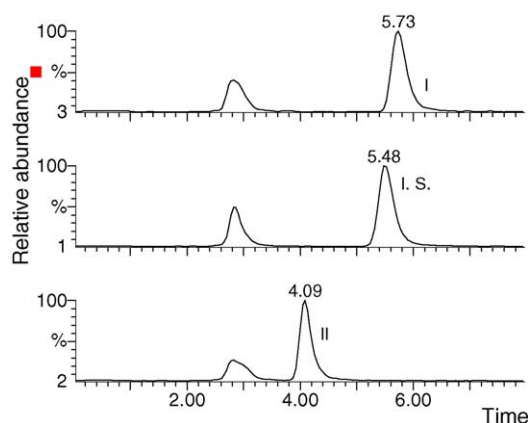


Fig. 3. SIM current profiles at m/z 790.7, 837.8 and 734.7 of the same plasma sample containing 600 ng/ml erythromycin propionate (I), 300 ng/ml roxithromycin (I.S.) and 600 ng/ml erythromycin base (II), with the retention time at 5.73, 5.48, 4.09 min, respectively.

3.3. Calibration curves

The concentration range covered was 2 ng/ml to 10 μ g/ml. Calibration curves ($y = ax + b$) were represented by plotting the peak area ratios (y) of the erythromycin propionate or base to I.S. roxithromycin versus the concentrations (x) of the calibration standards. Calibration curves were obtained from weighted ($1/x^2$) least-squares linear regression analysis of the data. The linearity was also assessed for consecutive days for the standard solutions of the same range of concentrations prepared from the stock solutions. The R.S.D. of intercept and slope for intra-day linearity is below 1.3%. The

R.S.D. of intercept and slope for intra-day linearity is below 2.5%. The calibration data are summarized in Table 1. The R.S.D. values for the intercept and slope are in agreement with the currently accepted USA Food and Drug Administration (FDA) bioanalytical method validation guidance [20]. The correlation coefficient was found to be over 0.997, indicating a good linearity.

3.4. Precision and accuracy

The precision of the method obtained by QC samples was evaluated by the inter- and intra-day ($n = 6$) assays at three different concentrations of erythromycin propionate and base in the range of 2 ng/ml–10 μ g/ml at levels corresponding to the lowest (2 ng/ml), near the middle (5 μ g/ml) and the highest (10 μ g/ml). The repeatability for inter-day and intra-day was below 4.2% R.S.D., 6.1% R.S.D., respectively.

The accuracy of the method obtained by QC samples was studied by calculating the mean recovery of the target compounds by adding standards known concentrations to the samples. The mean recovery was obtained by the determined concentrations as a percentage of the nominal concentrations. Every sample of the same concentration was injected at least six times. As a conclusion, the mean recovery for erythromycin base and propionate was 97.3–106.3% at the concentrations at levels corresponding to the lowest (2 ng/ml), near the middle (5 μ g/ml) and the highest (10 μ g/ml). As listed in Table 2, the precision and accuracy of the method met the acceptable criteria [20].

3.5. Lower limit of the quantitation

The analyte response at the LLOQ should be at least five times the response of blank baseline. The LLOQ was defined as the lowest concentration on the standard calibration curves with acceptable repeatability, recovery. The LLOQ of erythromycin propionate and base was 2 ng/ml. According to the USA FDA criteria [20], the analyte response at the limit of detection (LOD) should be reliably differentiated from background noise. The LOD of erythromycin base and propionate was 1 ng/ml.

3.6. Stability

The stability of the stock solutions was investigated by storing in the dark under refrigeration at -70°C . The stock solutions were discovered to be stable for at least 1 month

Table 1
Calibration data for erythromycin base and propionate added to drug-free plasma

Compound	Concentration range (ng/ml)	Slope	Intercept	Correlation coefficient
Erythromycin base	2–1000	0.001886	0.02103	0.9991
	1000–10,000	0.001338	0.25390	0.9973
Erythromycin propionate	2–1000	0.003126	0.01778	0.9995
	1000–10,000	0.002803	0.59870	0.9996

Table 2
Inter- and intra-day precision and accuracy of the method for determination of erythromycin base and propionate in human plasma

Measurement	Added (ng/ml)	Erythromycin base		Erythromycin propionate			
		Precision ^a	R.S.D. (%)	Mean accuracy ^b (n = 6) (%)	Precision ^a	R.S.D. (%)	Mean accuracy ^b (n = 6) (%)
Inter-day	2	3.5		103.9	4.2		101.0
	5000	2.8		99.0	2.0		100.0
	10,000	3.4		100.0	1.8		99.9
Intra-day	2	5.8		106.3	6.1		105.9
	5000	3.2		98.5	3.1		97.3
	10,000	2.1		103.2	2.8		101.1

^a Expressed as relative standard derivation.

^b Expressed as [(mean observed concentrations/nominal concentrations) × 100] (n = 6).

Table 3
Stability of erythromycin propionate (I) and base (II) in plasma QC samples

Stability test		Nominal concentration					
		2 ng/ml		5 µg/ml		10 µg/ml	
		I	II	I	II	I	II
Short-term stability (24 h)	Mean ± S.D.	1.9 ± 0.09	2.0 ± 0.11	5.03 ± 0.02	5.10 ± 0.01	10.03 ± 0.13	9.98 ± 0.15
	R.S.D. (%)	4.74	5.50	0.40	0.20	1.30	1.50
Freeze-thaw stability (3 cycles)	Mean ± S.D.	2.1 ± 0.13	1.9 ± 0.12	5.13 ± 0.08	4.97 ± 0.21	9.88 ± 0.23	10.05 ± 0.21
	R.S.D. (%)	6.19	6.32	1.56	4.23	2.33	2.09

n = 3 for short-term stability; n = 4 for freeze-thaw stability.

(<5% difference between fresh and test solution). The stability of erythromycin propionate and base at -70°C in human plasma was also assessed in QC samples after the storage at room temperature for 24 h, after three freeze and thaw cycles. The stability data of erythromycin propionate and base are summarized in Table 3. As Table 3 listed, no significant change in erythromycin propionate and base concentrations was found in human plasma samples stored at -70°C after three freeze-thaw cycles or at room temperature for 24 h.

3.7. Application

The described method was applied to a clinical trial. Typical plasma concentration–time profiles of erythromycin propionate and base obtained from one patient after the oral administration of one tablet of erythromycin stinoprate capsule (500 mg base equivalent) were shown in Fig. 4. The figure illustrated that the prodrug erythromycin propionate was rapidly absorbed and metabolized to the pharmacologically active metabolite erythromycin base in a short period.

4. Conclusion

The HPLC/MS method was developed and validated for simultaneous analysis of erythromycin propionate and its active metabolite, erythromycin base, in human plasma at the range of 2 ng/ml–10 µg/ml, providing necessary precision, accuracy and sensitivity according to USA FDA criteria [20]. Simple sample pre-procedure and short run time is suitable for large sample batches. The method proved to be suitable for assessing the bioavailability and pharmacokinetic characterization of erythromycin stinoprate.

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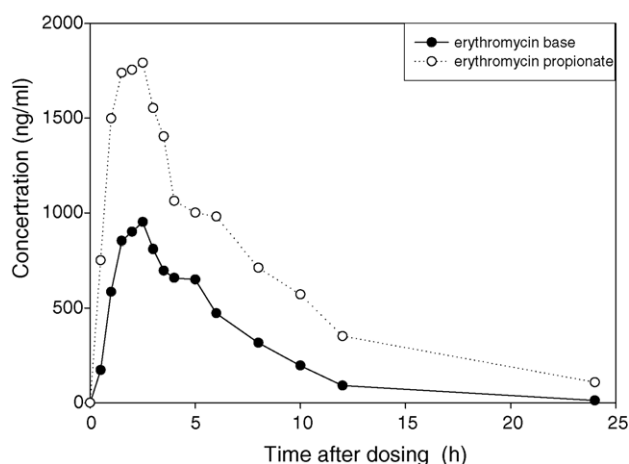


Fig. 4. Concentrations (ng/ml) of erythromycin base and erythromycin propionate in plasma of one patient after oral administration of one tablet of erythromycin stinoprate capsule (500 mg base equivalent).

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