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Simultaneous determination of erythromycin ethylsuccinate and its metabolite erythromycin in human plasma using liquid chromatography–electrospray ionization mass spectrometry for clinical study

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Abstract

A sensitive and selective liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) method was developed for simultaneous identification and quantification of erythromycin ethylsuccinate and erythromycin in human plasma, which can be well applied to clinical study. The method was based on liquid–liquid extraction, followed by a LC procedure with an ODS C18 column, and mixture of acetonitrile and 1.67 mmol/l acetic acid as mobile phase. MS detection was performed using a single quadrupole mass spectrometer in positive selected ion monitoring (SIM) mode. The method was validated to be linear, precise and accurate. The lower limit of quantification of erythromycin ethylsuccinate and erythromycin were both 0.5 ng/ml. The proposed method enables the unambiguous identification and quantification of erythromycin ethylsuccinate and erythromycin for clinical drug monitoring.

Keywords: Erythromycin ethylsuccinate; Erythromycin; LC–ESI-MS; Human plasma

1. Introduction

Erythromycin ethylsuccinate (EMES), a prodrug of erythromycin (EM), belongs to macrolide antibiotics which are still the footstones of antibacterial therapy even after five decades of use. As an ester of EM, EMES which is antibacterially inactive itself achieves the effects after it is hydrolyzed to EM in plasma and body fluid [1]. EMES is acid-resistant and has less gastrointestinal side effects. Though many new macrolide antimicrobials are developed and marketed recently, EMES is still widely used throughout the world [2–4]. The satisfying effect–price ratio makes it very popular in developing countries. In China, the usage frequency of EMES ranks the third in antibiotics, posterior to amoxicillin and cefalexin. It is applied as therapy especially for upper and lower respiratory tract infections of suspected bacterial aetiology and in patients who are allergic to penicillin and cephalosporins [5]. The C_{max} of EMES in human plasma reported was rather low for clinical study, which was about 1 μg/ml after administrating a dosage of 600 mg [6], 500 mg [4,7] or 750 mg [8] orally.

Few studies have been reported for the simultaneous determination in plasma of the inactive intact prodrug and its hydrolyzed active product, the EM base, due to the lack of suitable analytical methods. Microbiological analytical method has been widely used [4,7–10]. But it suffers a poor specificity and a relatively high limit of detection. The analytical chemistry techniques applied to macrolides before 1998 was already reviewed by Kanfer [11]. However few were focused on plasma samples. Recently, high performance liquid chromatography (HPLC) is still a main method for analyzing EM and its derivatives [6,12–16]. To overcome the problem that EM has a low molar absorptivity because of its lack of a suitable chromophore, a short wavelength (about 215 nm) is always used, which often encounters the trouble of many interfering components at the adjacent wavelength in plasma. HPLC with fluorescent detection was often criticized for complex operation of pre- or post-column

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derivatization [17]. More recently, liquid chromatography mass spectrometry (LC–MS) method was widely used for drug analysis. Its application in analysis of antibiotics was summarized by Niessen [18]. However, this review showed LC–MS method was mostly applied to the residue analysis for macrolides and few were utilized for plasma analysis. A phase-system switching continuous-flow fast atom bombardment mass spectrometry (FAB-MS) had been used for quantification of EMES in human plasma [1]. This technique needed a flow split via the LC–MS interface, which might decrease the sensitivity and reproducibility, and the ionization mode may cause serious damages to the analyte structures. A newly published literature [19] focused on the simultaneous analysis of erythromycin propionate and base in human plasma using LC–MS. Unfortunately, this assay also needed the flow split and the deproteinization of sample preparation seemed not enough, which might be unsuitable for MS analyzer. For clinical purpose, a time-saving procedure is also important. Therefore, a simple, rapid, sensitive and economical method of simultaneous determination of EMES and EM is developed here for clinical drug concentration monitoring and curative-effect evaluation.

2. Experimental

2.1. Reagents and chemicals

Acetonitrile of HPLC grade was obtained from Fisher Chemicals (USA). Acetic acid, diethyl ether and anhydrous sodium carbonate used were commercially available and all of analytical grade. Water was purified by filtrating through a Milli-Q system (Millipore, Bedford, MA, USA) before use. The standard products of EMES (purity ≥98.2%), EM (purity ≥99.0%) and internal standard (IS) diazepam (purity ≥99.6%) were provided by National Institute for the Control of Pharmaceutical and Biological Products (China). Stock solutions were both prepared at 1 mg/ml in acetonitrile and stored at −20 °C in the dark (Fig. 1).

2.2. Apparatus and chromatographic conditions

A Shimadzu 2010A LC–MS system with electrospray ionization (ESI) interface, and Shimadzu LCMS solution workstation software (Version 2.02) for the data processing, were utilized to perform the analytical procedures. The system consisted of two Shimadzu LC-10ADvp pumps, a Shimadzu SIL-HTc autosampler, a Shimadzu CTO-10Avp column oven and a Shimadzu DGU-14AM online degasser. A Q-array-Octapole-Quadrupole mass analyzer was used as the detector. The LC process was carried out on an ODS column (250 mm × 2.0 mm i.d., 5 μm particle size; Shimadzu). The mobile phase, at the flow rate of 0.2 ml/min, was made up of acetonitrile and water containing 1.67 mmol/l acetic acid (70:30, v/v). Oven temperature was kept at 40 °C. The optimized MS parameters were selected as followed: CDL (curve desolvation line) temperature: 250 °C, the block temperature: 200 °C, the probe temperature: 250 °C; detector voltage: 1.4 kV, probe voltage: 4.5 kV, CDL voltage: 30 V, Q-array dc voltage: 0 V, rf voltage: 150 V. Nitrogen, supplied by the Gas supplier center of Nanjing University, China, served as nebulizer gas (flow rate: 1.5 l/min) and curtain gas (pressure: 1 MPa). Mass spectra were obtained at a dwell time of 0.2 and 1 s for SIM and scan mode, accordingly. The MS acquisition was performed under positive selected-ion monitoring (SIM) mode.

2.3. Sample preparation

0.2 ml plasma and 10 μl of IS working solution (1 μg/ml) were pipetted into 15 ml plastic stoppered conical extraction tubes. After vortex mixing for 30 s, 25 μl of 0.1 mol/l Na_2CO_3 water solution were added, and then followed by another 30 s vibration. After 5 ml diethyl ether was transferred inward, the tubes were stoppered well and shaken vigorously for 1 min. Following centrifugation at 1200 × *g* for 5 min, 4 ml of the upper organic layer was transferred out and evaporated to dryness at 50 °C under a gentle stream of nitrogen. The residue was reconstituted with 200 μl acetonitrile and centrifuged at 21 000 × *g*

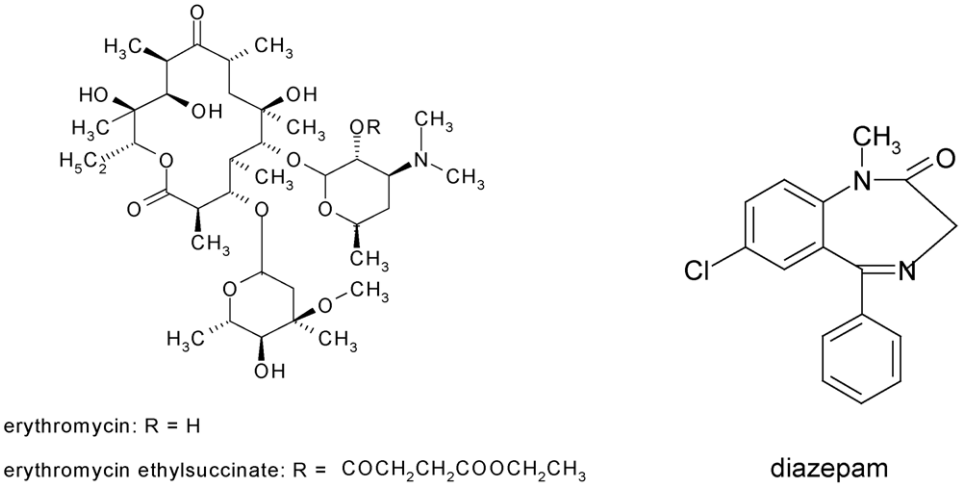

Fig. 1. The structures of erythromycin ethylsuccinate, erythromycin and diazepam.

at 4 °C for 10 min. The supernatant (80 μl) was pipetted to an autosampler vial, and 5 μl was injected onto column for analysis.

Quality control (QC) samples (prepared by the research principal) and calibration curve samples were made by spiked blank human plasma with 10 μl of appropriate standard solutions to requisite plasma concentrations, followed by the same operation listed upward.

2.4. Method validation

Specificity was ascertained by analyzing six blank human plasma samples without adding IS to determine the interference with the analytes. Six sets of calibration curves ranging from 0.5 to 5000 ng/ml for EMES and EM, respectively, were constructed by plotting the peak-area ratios of target/IS versus analyte concentrations in blank human plasma on a single day. Within-day and between-day precisions were investigated by reanalyzing QC samples at low, medium and high concentrations, which were determined by calibration curve prepared on the same day, for five times on a single day and once on five consecutive days. Accuracy of the method was determined by the relative error (R.E.), which was calculated by the equation: (mean of determined concentration − actual concentration)/actual concentration × 100%, and precision was evaluated by the relative standard deviations (R.S.D.). Samples for recovery study (10, 100, 1000 ng/ml) were built by adding the standards to blank human plasma and following the common operations mentioned before, while the reference samples were relative standards prepared in acetonitrile. Only peak areas of EMES or EM were compared at three levels of concentration to provide recovery values.

2.5. Stability study

Within-run stability was tested by reanalyzing QC samples at three different concentrations levels kept under the autosampler conditions (4 °C) ever and again till the end in every routine analysis. The amount of QC samples was at least 5% of the total samples. Long-term and freeze–thaw stability were together evaluated by analyzing QC samples at low, medium and high concentrations which were stored at −20 °C for a whole week (far exceeded the time between the date of first sample collection and the date of last sample analysis) and then followed by three freeze–thaw cycles, each of which contained a storage at −20 °C for 24 h and thaw at 37 °C.

2.6. Clinical application

The assay was applied to clinical drug concentration monitoring. According to the trial protocol, four Chinese male volunteers involved, aged 22–25, who were identified as healthy based on medical history, physical examination, and electrocardiography (ECG) were eligible for inclusion, and none of exclusion criteria for any clinically relevant laboratory abnormalities of clinical chemistry and hematology. Volunteers all signed a statement concerning informed consent of their own accords after apprised of the content and risk of the trial in detail.

Volunteers were administrated EMES at a dosage of 500 mg/person orally, and kept in the clinical cure room till the end of trial with free but mild activities. Blood samples were taken prior to and at 10, 20, 30 min, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36 h after drug ingestion, using heparin as anticoagulant. After the centrifugation, plasma was transferred out and then extracted for analysis as immediately as possible. In every turn of clinical sample analysis, a corresponding standard curve was made to calculate the drug concentration.

3. Results and discussion

3.1. Identification of analytes and chromatography

The total positive ion spectra scanned from m/z 300 to 1000 of EMES and EM of standard samples respectively are shown in Fig. 2. The major ions observed were $[M + H]^+$, $m/z = 862.75$; $[M + Na]^+$, $m/z = 884.75$; $[M + K]^+$, $m/z = 900.75$ for EMES and $[M + H]^+$, $m/z = 734.60$; $[M + Na]^+$, $m/z = 756.60$ for EM. It was surmised that Na^+ and K^+ prevalently in the water produce ions of $[M + Na]^+$ and $[M + K]^+$, whereas extra acetic acid supplement in the mobile phase made protonated molecular ions $[M + H]^+$ predominant with robust signal. Thus, the ions of $[M + H]^+$, $m/z = 862.75$ for EMES and $[M + H]^+$, $m/z = 734.69$ for EM were chosen for monitoring under the SIM mode.

Under the acquisition of SIM mode, a typical chromatogram of an extract from a volunteer's plasma gave peaks not presented in the chromatogram of blank sample (Fig. 3). The representative peaks had the same m/z values as those from standard samples of EMES and EM. The IS was also monitored in protonated molecular ion form $[M + H]^+$, $m/z = 285.00$, because of its acceptable effect of protonation under such MS conditions. It is clear that blank human plasma yielded relative clean chromatograms without significant interfering peaks. The retention times of EMES, EM and diazepam were about 2.5, 2.3 and 5.5 min, respectively.

3.2. Liquid–liquid extraction

Liquid–liquid extraction is easy and economical. The macrocyclic lactone ring of macrolides has a good solubility in organic solvent. The molecules often show alkaline because of dimethyl amidogen sugars attached to the ring. So addition of Na_2CO_3 during extraction makes the analytes in molecular form, which can bring relative better extraction efficiency, and reduces interference since most endogenous compounds are of acidic nature. The quantity of Na_2CO_3 added has been optimized for a weak alkaline condition to avoid the ester's hydrolysis of EMES in high pH conditions.

3.3. Advantages of LC–ESI-MS

LC–ESI-MS has several advantages for the analysis of EMES in plasma. Theoretically, analytes that cannot be separated well by LC also can be analyzed by MS, as long as they have different mass–charge ratios. The combination of LC with ESI-MS leads to a relative short run time, comparing with those methods developed before, and yields both high selectivity and sensitivity. ESI

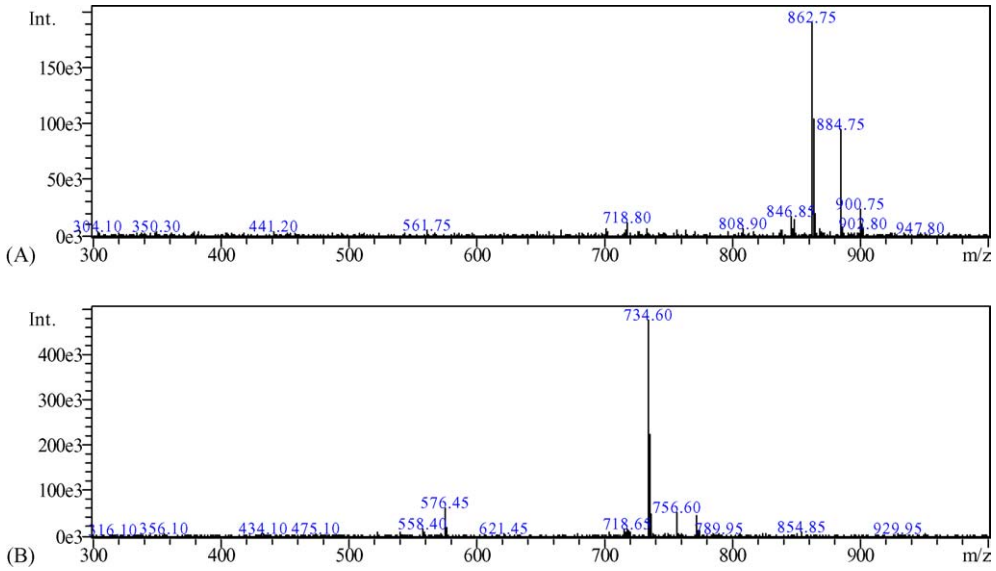

Fig. 2. Positive ion electrospray mass spectrum obtained in scan mode from standard samples of erythromycin ethylsuccinate (A) and erythromycin (B), respectively, with abundance of $[M+H]^+$.

is a "gentle" ionization technique that produces high mass-to-charge $[M+H]^+$ precursor ions with minimal fragmentation of the analytes, which is more suitable for clinical pharmacokinetic study than FAB-MS [1].

3.4. Method validation

Six series of calibration samples with concentrations of 0.5, 1, 5, 10, 50, 100, 500, 1000, 5000 ng/ml both for EMES

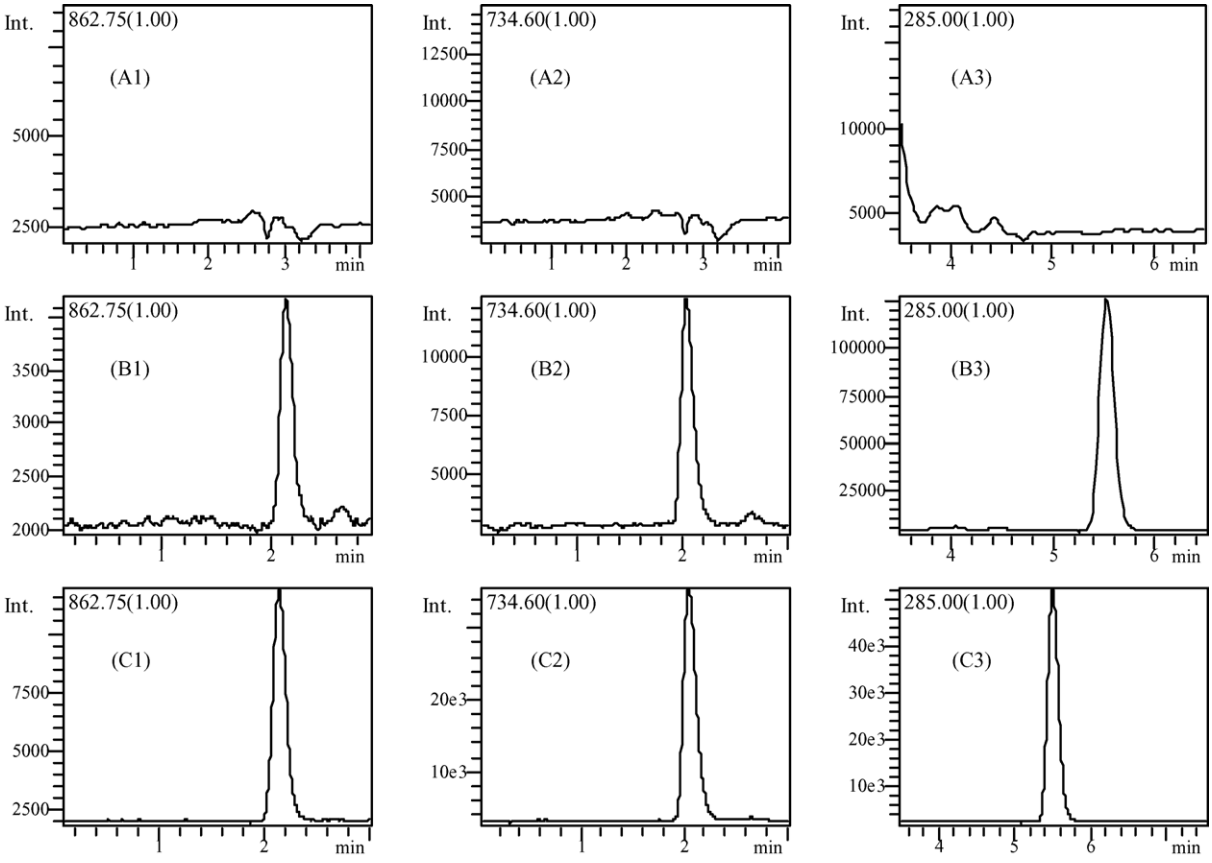

Fig. 3. Mass chromatograms of analytes under SIM mode from human plasma: (A) blank human plasma, (B) blank human plasma spiked with erythromycin ethylsuccinate (100 ng/ml), erythromycin (100 ng/ml) and internal standard, respectively, (C) plasma sample 1 h after oral administration of erythromycin ethylsuccinate; (1), (2) and (3) represent the monitored ions of erythromycin ethylsuccinate, erythromycin and diazepam separately in the SIM mode.

Table 1
Evaluation of low limit of quantification (LLOQ) of erythromycin ethylsuccinate and erythromycin in blank human plasma ($n = 6$)

Compound		n						Mean	S.D.	R.S.D.
		1	2	3	4	5	6			
	Spiked concentration (ng/ml)	0.5						–	–	–
Erythromycin ethylsuccinate	Measured concentration (ng/ml)	0.41	0.60	0.40	0.49	0.54	0.41	0.48	0.08	17.40
	Accuracy (%)	82.00	120.00	80.00	98.00	108.00	82.00	95.00	16.53	17.40
Erythromycin	Measured concentration (ng/ml)	0.43	0.57	0.59	0.45	0.44	0.41	0.48	0.08	16.10
	Accuracy (%)	86.00	114.00	118.00	90.00	88.00	82.00	96.33	15.51	16.10

Accuracy (%): measured concentration/spiked concentration × 100%.

Table 2
Method precision and accuracy in blank human plasma ($n = 5$)

Compound	Spiked concentration (ng/ml)	Within-day			Between-day		
		Measured concentration (mean ± S.D., ng/ml)	R.E. (%)	R.S.D. (%)	Measured concentration (mean ± S.D., ng/ml)	R.E. (%)	R.S.D. (%)
Erythromycin ethylsuccinate	10	10.1 ± 1.2	1.0	11.9	9.4 ± 0.2	−6.0	2.1
	100	95.7 ± 3.6	−4.3	3.8	97.3 ± 1.1	−2.7	1.1
	1000	989.7 ± 52.6	−1.0	5.3	976.5 ± 7.9	−2.4	0.8
Erythromycin	10	10.0 ± 0.9	0	9.3	10.2 ± 0.6	2.0	5.9
	100	100.7 ± 4.0	0.7	4.0	102.4 ± 3.0	2.4	2.9
	1000	994.2 ± 27.4	−0.6	2.8	1008.5 ± 14.8	0.9	1.5

and EM were analyzed to test the linearity of the method. Results showed linearity from 0.5 to 5000 ng/ml for EMES and from 0.5 to 1000 ng/ml for EM. Non-weighted linear regression of the mean value gave the following respective equations: EMES, $y = 9 \times 10^{-5}x + 4.95 \times 10^{-5}$ ($r^2 = 0.988$); EM, $y = 1 \times 10^{-3}x + 7.61 \times 10^{-4}$ ($r^2 = 0.998$), where y represented the peak-area ratio and x was the concentration. The lower limit of quantification (LLOQ) of EMES and EM were both 0.5 ng/ml by determining the aliquots of nominal concentration ($n = 6$) and six different blank human plasma, yielding a detector response as about 10 times as the detector noise, with a precision of <20% and accuracy of 80–120% (Table 1). Analytical accuracy and precision data were shown in Table 2. Within-day and between-day performances, expressed as RSD, were less than 15% at all concentrations within the standards. The recovery of EMES was found to be 56 ± 3% (recovery ± standard deviation (S.D.), $n = 3$) at 10 ng/ml level, 59 ± 4% ($n = 3$) at 100 ng/ml, and 58 ± 7% ($n = 3$) at 1000 ng/ml. The recovery of EM was 82 ± 3% ($n = 3$) at 10 ng/ml level, 77 ± 4% ($n = 3$) at 100 ng/ml, and 77 ± 7% ($n = 3$) at 1000 ng/ml, respectively. These results indicated the liquid–liquid extraction efficiency was well acceptable.

3.5. Stability study

Stability results shown in Table 3 illuminated the analytes were stable for at least 48 h (about the total analysis time for one turn of the trial) at the autosampler's conditions (4 °C) after

Table 3
Results of stability study ($n = 5$)

Compound	Spiked concentration (ng/ml)	Within-run stability			Long-term and freeze–thaw stability		
		Measured concentration (mean ± S.D., ng/ml)	R.E. (%)	R.S.D. (%)	Measured concentration (mean ± S.D., ng/ml)	R.E. (%)	R.S.D. (%)
Erythromycin ethylsuccinate	10	10.0 ± 0.6	0	5.7	7.8 ± 0.8	−22.0	9.7
	100	101.6 ± 2.4	1.6	2.4	76.5 ± 9.4	−23.5	12.3
	1000	988.1 ± 6.8	−1.2	0.7	817.9 ± 39.5	−18.2	4.8
Erythromycin	10	9.8 ± 0.5	−2.0	5.5	9.1 ± 0.6	−9.0	6.6
	100	99.3 ± 1.2	−0.7	1.2	91.3 ± 4.7	−8.7	5.1
	1000	943.9 ± 40.3	−5.6	4.3	901.6 ± 64.2	−9.8	7.1

extraction. But storing at −20 °C for a whole week followed by three freeze–thaw cycles seemed to affect the stability of EMES at a certain extent.

Hydrolysis half-life of EMES in human plasma at 37 °C was reported to be 60 min at 5 mg/l level with a conclusion that EMES hydrolyzed more rapidly than acetyl and propionyl esters of EM [20]. Another report of the half-lives of EMES in human plasma were 181.6 and 55.2 min at 4 and 37 °C, respectively [6]. Tsuji also claimed approximately 2% hydrolysis of EMES took place during the 4 h of the freezing process and 10% after storage for 13 days at −20 °C [17]. Hence, he suggested an immediate analysis was urgently needed after sampling likewise. Besides, for the method validation study, the long-term stability test in plasma was actually lacked or avoided in some important reports [1,6,19].

These phenomenons seemed to support our suggestion that EMES was not very stable for a long-term storage. Therefore, in order to eliminate the influence of hydrolysis in plasma as possible as it can, samples must be analyzed immediately. In our studies, the immediate analysis was furthermore required by the clinical purpose. The within-run stability results appeared to prove the relative reliance of the short analysis procedure.

3.6. Avoidance of degradation of EMES to EM: some measurements and proofs

Stability study showed EMES was not very suitable in plasma for a long term. Therefore some measurements must be taken to avoid further degradation in extraction procedure and MS analysis. As shown in Fig. 2, there were no typical ions of EM in the scan spectrum of EMES (standards), which partly indicated that the ester bond of ethylsuccinate could not be broken in the gentle ionization source and no in-source fragmentation displayed. So with the ESI source, an immediate extraction by diethyl ether and a suitable pH environment, degradation of the ester during analysis procedure was minimized under current conditions. However, a thimbleful of EM was actually detected under SIM mode from reference samples spiked with only EMES (data not shown). But by reanalyzing these samples, the amount did not increase as time passed; moreover, the ratio of EMES and EM was very similar to that of the standard solution of EMES analyzed under the same conditions. By the way, SIM mode has much higher sensitivity than scan mode for single quadrupole mass spectrometer, which may be why no characteristic ions of the thimbleful of EM was displayed in the scan spectrum of EMES, opposite to the SIM circumstances. It was suggested the thimbleful of EM detected did not came from ester's degradation during the analysis procedure, but from the standard product we purchased.

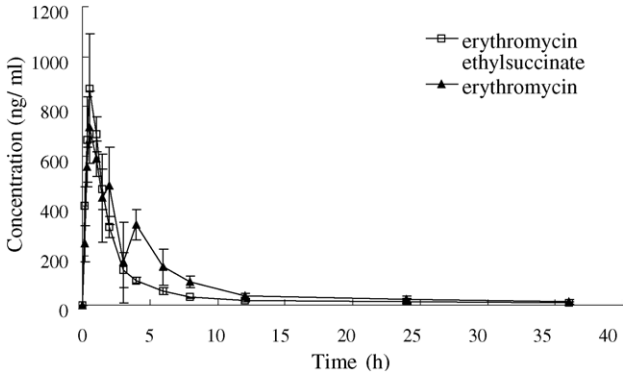

Fig. 4. Mean plasma concentration vs. time profiles in four healthy volunteers after oral administration of single 500 mg dose of erythromycin ethylsuccinate.

3.7. Matrix effects

Matrix effects from co-eluting endogenous substances provide possible source of problems regarding assay interference and ion signal repression, although matrix-matched calibration standards were used. In chromatographic separation, the retention times of analytes were prolonged behind the dead time (t_0) of the column (about 1.5 min) to avoid main endogenous interferences, which were mostly eluted before or around t_0. Moreover, ion suppression effect was evaluated by comparing the peak areas of EMES (100 ng/ml) and EM (50 ng/ml) in six QC samples with those of standard solutions that had been prepared in the same way as the QC samples except that water was substituted for drug-free plasma. For EMES and EM, the mean peak areas from the six QC samples had relative errors of 4.1 and 4.4%, respectively, when compared with that for these standard solutions. These observations indicated that no endogenous substances significantly influenced the ionization of these analytes.

3.8. Clinical application

Clinical drug monitor after oral administration of EMES was actualized by determining volunteers' plasma concentrations using the method established here. Fig. 4 represents mean plasma concentration–time profiles of EMES and its metabolite EM after a single dose (500 mg) of EMES in four volunteers. From the pharmacokinetic profiles, we found EMES was eliminated fast in human bodies, which accorded with its "prodrug mechanism". EM maintained a relative high level of plasma

Table 4
Pharmacokinetic parameters after oral administration of a single dose of erythromycin ethylsuccinate (500 mg) to Asian volunteers, calculated by statistical moment analysis using DAS Version 1.0. ($n = 4$)

Parameters	AUC_{0-36} (μg/l h)	$AUC_{0-\infty}$ (μg/l h)	MRT_{0-36} (h)	MRT (h)	T_{max} (h)	C_{max} (ng/ml)
Erythromycin ethylsuccinate	2179.56	2465.55	5.65	9.98	0.50	872.88
Erythromycin	3159.80	3394.35	6.81	12.44	0.50	714.97

concentration. Two-peak phenomenon appeared in the curve of EM. Examination of the second peak revealed that it occurred close to volunteers' midday meals, suggesting biliary recycling may be the cause. This phenomenon in human was also reported by Croteau [21]. Pharmacokinetic parameters, listed in Table 4, were calculated by statistical moment analysis using DAS software (Version 1.0, Medical Institute of Wannan, China).

Only 0.2 ml plasma is needed in this method illustrates the high sensitivity. So no more than 1 ml of whole blood were taken from the volunteers in our trial, which foreshows a clinical application of decreased suffer and increased acclimation to patients. Furthermore, if more blood is sampled, the rest can be used for other clinical purposes simultaneously.

4. Conclusion

A sensitive and selective LC–ESI-MS method for simultaneous qualification of erythromycin ethylsuccinate and its metabolite erythromycin in human plasma is established and validated to be linear, accurate and precise. The lower limit of quantification is significant, more sensitive than any other ever reported. Simple liquid–liquid extraction procedure and short run time can curtail test's cost and time that is very important for large clinical sample batches. It is indicated that this method is suitable for the analysis of erythromycin ethylsuccinate and erythromycin in human plasma samples collected for clinical studies.

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