A Robust, Simple and Rapid Validated Method for Estimation of Erythromycin Ethylsuccinate in Human Plasma by Liquid Chromatography-tandem Mass Spectrometry and its Application to Clinical Study

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Abstract: A rapid, simple and selective method for estimation of erythromycin ethylsuccinate in human plasma was validated using imipramine as internal standard. The analyte and internal standard were extracted from plasma using simple solid phase extraction. The compound were separated on a reverse-phase column with an isocratic mobile phase consisting of 0.1% formic acid in water and acetonitrile (20:80, v/v) and detected by tandem mass spectrometry in positive ion mode. The ion transition recorded in multiple reaction monitoring mode were m/z 862.5 → 286.2 for erythromycin ethylsuccinate and m/z 281.2 → 208.1 for internal standard. Linearity in plasma was observed over the concentration range 10 – 1500 ng/mL for erythromycin ethylsuccinate. The method was validated to be linear, precise and accurate. The lower limit of quantification of erythromycin ethylsuccinate was 10.0 ng/mL. The mean recovery for erythromycin ethylsuccinate was 62.7%. The coefficient of variation of the assay was less than 7.0 % and accuracy of 91.4% to 97.1%. The validated method was applied to pharmacokinetic study of erythromycin oral suspension 250 mg/mL (as ethylsuccinate) in healthy human volunteers.

Key words: Erythromycin ethylsuccinate; Imipramine; LC-MS-MS; human plasma

INTRODUCTION

Erythromycin ethylsuccinate (EES) is chemically known as erythromycin 2'-ethylsuccinate) (Figure 1a). It is a prodrug of erythromycin, belongs to macrolide antibiotics which are still the footstones of antibacterial therapy. As an ester of erythromycin, EES is antibacterially inactive itself achieves the effects after it hydrolysed to erythromycin in body fluid (Kokkonen 1991). EES is acid resistant and has less gastrointestinal side effects. Though many macrolide antimicrobials are developed and marketed recently, EES is widely used (Change 2001; Strachounski 2001; Min 2004). It is applied as a therapy especially for upper and lower respiratory tract infections of suspected bacterial aetiology and in patients who are allergic to penicillin and cephalosporin (Pinchichero 1998). A C_max of about 1µg/mL for EES in human plasma was reported for clinical study with an oral dose of 600 mg (Croteau 1987), 500 mg (Min 2004; Chu 2000) and 750 mg (Qiao 1998).

Microbiological analytical method has been reported for simultaneous determination of EES and its hydrolysed form, erythromycin (Min 2004; Chu 2000; Qiao 1998; Bell 1969; Bennett 1966). But it suffers a poor specificity and a relatively high limit of detection. HPLC methods for estimation of erythromycin and its derivative have been reported (Croteau 1987; Wang 2000; Huebra 2004; Leal 2001; Dehouck 2003; Lingerfelt 1999), which often encountered interference at the adjacent wavelength in plasma. A phase–system switching continuous-flow fast atom bombardment mass spectrometry (FAB-MS) has been used for quantification of EES in human plasma (Kokkonen 1991). This technique needed a flow split via the LC-MS interface, which might decrease the sensitivity of the method. A LC-MS method for simultaneous estimation of erythromycin propionate and base in human plasma have been reported which involves protein precipitation in sample preparation. LC-MS/MS method for estimation of EES and its metabolite have been reported which involved liquid–liquid extraction in sample preparation. This method required immediate samples analysis after clinical conduction due to stability issue of EES in plasma. A run time of about 7 minutes reported in this method (Gu 2006).

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In the present study a robust, simple and rapid method have been developed and validated to estimate EES in human plasma, with a total run time of 3 min. The method involves simple solid phase extraction which allows for a high throughput of over 150 samples per day. Solid phase extraction technique has been applied which ensured a lot more sample clean up resulting in better selectivity and consistent recovery. EES was stabilized in plasma using acidic condition. Acidic condition also maintained at the time of sample collection in clinical phase. Imipramine (Figure 1b) was used as an internal standard.

**EXPERIMENTAL**

**Materials and Chemicals**

A USP Reference standard procured for Erythromycin ethylsuccinate and Imipramine was obtained from Sigma-Aldrich. HPLC grade methanol and acetonitrile were purchased from J. T. Baker INC (Phillipsburg, NJ, USA). Formic acid of AR grade was procured from Merck Ltd (Mumbai, India). Phenomenex Strata-X (30mg, 1mL) SPE cartridges were procured from Spincotech Pvt. Ltd., (India). Water used in the entire analysis was prepared through Milli-Q water purification system from Millipore (Bangalore, India). Blank human plasma with Na Heparin as anticoagulant was used for preparation of calibration and quality control samples.

**Liquid Chromatography and Mass Spectrometric Conditions**

The liquid chromatography system (Shimadzu, Kyoto, Japan) coupled with mass spectrometer API-2000 (AB, Sciex, Canada) used. The analytical column, Hypurity Advance (100 mm x 4.6 mm, 5 mm particle size) from Thermo Electron Corporation (Cheshire, UK) was used for separation of analyte and internal standard. Mobile phase of 0.1% formic acid in water (v/v) with acetonitrile in ratio of 20:80 (v/v) was pumped isocratically at flow rate of 0.4 µL/min. Auto sampler temperature was set at 4 °C and the injection volume was 5µL. The column oven temperature was maintained at 40 °C and the total LC run time was 3.0 min.

The API-2000 (AB Sciex, Canada) LC-MS-MS apparatus was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ion m/z 862.5 to the product ion m/z 286.2 for erythromycin ethylsuccinate and the transition of the protonated molecular ion m/z 281.2 to the product ion m/z 208.1 for the internal standard, imipramine. The instrument response was optimized for erythromycin ethylsuccinate and imipramine by infusing a constant flow of a solution of the drug dissolved in mobile phase.

Electrospray ionization (ESI) was performed in the positive ion mode. The source temperature was set 400°C. Ion spray voltage of 2000 V was used. Nitrogen was used as the collision gas. The Curtain gas was set 20 and Gas 1 and Gas 2 were optimized as 50 and 55 respectively. Compound dependant parameters set for erythromycin ethylsuconitate and IS were DP voltage: 18 and 17 V; FP voltage: 400 and 300 V; CXP: 12 and 13 V; EP: 10 and 7; collision energy: 42 and 30 eV respectively. Q1 and Q3 were maintained at unit resolution and the dwell time was kept at 200ms. The collision activated dissociation (CAD) gas pressure was set at 4 (arbitrary unit). The instrument was interfaced with a computer running analyst 1.4.2 software.

**Preparation of Standards and Quality Control Samples**

A 1mg /mL stock solution for EES and Imipramine were prepared by dissolving their accurately weighted compounds in acetonitrile and methanol respectively. Two separate stock solutions of EES were prepared for bulk spiking of calibration curve and quality control samples for the method validation experiment as well as the subject sample analysis. The stock solution of EES thus prepared was serially diluted to prepare working solution in required concentration range with acetonitrile. All stock dilutions of EES performed with acetonitrile to avoid any hydrolysis of EES. The calibration standards and quality control (QC) samples were prepared by spiking (5% of the total plasma volume) with working solutions. Before spiking drug in plasma, a 30 µl of 2.5% ortho phosphoric acid in 50mM Di Sodium hydrogen Phosphate solution is added to each 0.285 mL of blank plasma to avoid hydrolysis of EES. Calibration standards were prepared at concentration of 10.0, 20.0, 50.0, 150.0, 300.0, 600.0, 900.0, 1200.0, and 1500.0 ng /mL for EES. Similarly, quality control standards (QC’s) were prepared at four different concentrations namely, 10.2 (LLOQ), 28.0 (LQC), 475.0 (MQC) and 1050.0 (HQC) ng /mL. Sufficient calibration standards and quality control standards were prepared to validate the method and to serve as standards and controls during the assay of all study
samples. However during the study, only three levels of controls were prepared as LQC (Lower Quality Control), MQC (Middle Quality Control) and HQC (Higher Quality Control). Aliquots of the standards and quality controls were stored together with the study samples at -70 °C until used for sample processing. Study samples were also added 2.5% ortho phosphoric acid in 50mM Di Sodium hydrogen Phosphate solution before storage.

**Extraction Procedure**

The plasma samples (330 µl) were transferred to 1.7-mL clear tubes (Tarsons, India) added 25µl of internal standard (working solution of 5 µg /mL of Imipramine). The samples were vortexed to mix for 30 sec. and centrifuged for 5 minutes at 14000 rpm. After centrifugation the samples were loaded on phenomenon strata-X 30mg/1mL cartridge pre-conditioned with 1 mL methanol followed by 1 mL 0.1% (v/v) formic acid in water. The plasma matrix was drained out from the extraction cartridges by applying positive nitrogen pressure. The extraction cartridges washed with 2 mL of 0.1% (v/v) formic acid in water. The analyte and the internal standard were eluted with 1.0 mL of acetonitrile. A 5 µL of the eluant was injected.

**Method Validation**

**Selectivity.** Selectivity was performed using 10 different sources of blank plasma comprising of 6 normal, two haemolysed and two lipemic. They were processed as per the extraction method and their response was assessed at the retention time of analytes and the internal standard with six LLOQ samples for erythromycin ethylsuccinate were prepared from the screened blank plasma samples which had the least interference.

**Carry over.** Carryover effect was evaluated to ensure that the rinsing solution used to clean the injection needle and port is able to avoid any carry forward of injected sample in subsequent runs. The design of the experiment comprised blank plasma, LLOQ, upper limit of quantitation (ULOQ) followed by blank plasma to check for any possible interference due to carryover.

**Linearity and lower limit of quantification.** The linearity of the method was determined by analysis of five standard plots associated with a nine-point standard calibration curve. The ratio of area response for analyte to IS was used for regression analysis. Each calibration curve was analyzed individually by using least square weighted (1/x²) linear regression. The calculation was based on the peak area ratio of analyte versus the area of internal standard. The concentration of the analyte were calculated from calibration curve (y = mx + c; where y is the peak area ratio) using linear regression analysis with reciprocate of the drug concentration as a weighing factor (1/concentration²). Several regression types were tested and the linear regression (weighted with 1/concentration²) was found to be the simplest regression, giving the best results (r² ≥ 0.9990). The lowest standard on the calibration curve was accepted as the lower limit of quantitation (LLOQ), if the analyte response was at least five times more than that of drug free (blank) extracted plasma. The deviation of standards other than LLOQ from the nominal concentration should not be more than ±15.0% for LLOQ it should not be more than ± 20.0%.

**Accuracy and precision.** The intra-batch and inter-batch accuracy and precision were determined by replicate analysis of the four quality control levels on three different days. In each of the precision and accuracy batches, six replicates at each quality control level were analysed. Mean and standard deviation (SD) were obtained for calculated drug concentration level were analysed. Mean and standard deviation (SD) were obtained for calculated drug concentration over these batches. Accuracy and precision were calculated in terms of relative error (%RE) and coefficient of variation (% CV) respectively.

**Matrix effect.** The assessment of matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionization) was performed by processing six lots of different normal controlled plasma samples in replicate (n=4). LQC and HQC working solutions were spiked post extraction in duplicate for each lot. The results found were well within the acceptable limit set i.e. the RSD of area ratio to be within ± 15% at each level tested.

**Recovery.** Absolute recoveries of the analyte were determined at the three different quality control levels viz. LQC, MQC and HQC, by comparing the peak areas of the extracted plasma samples with those of the unextracted standard mixtures (prepared in the elution solution at the same concentrations as the extracted samples) representing 100% recovery.

**Dilution integrity.** The dilution integrity experiment was intended to validate the dilution test to be carried out on higher analyte concentrations (above ULOQ), which may be encountered during real subject samples analysis. It was performed at 1.6 times the ULOQ concentration. Six replicates samples of ½ and ¼th concentration were prepared and their concentrations were calculated by applying the
dilution factor of 2 and 4 respectively against the freshly prepared calibration curve.

**Stability.** All stability results were evaluated by measuring the area response (analyte/IS) of stability samples against comparison samples of identical concentration. Stock solutions of EES and IS were checked for short term stability at room temperature and long term stability at 2-8°C. The solutions were considered stable if the deviation from nominal value was within ±10.0%. Bench top stability, autosampler stability (process stability), freeze thaw stability, and long-term stability in plasma were performed at LQC and HQC level using six replicates at each level. Freeze-thaw stability was evaluated by successive cycles of freezing (at -70°C) and thawing (without warming) at room temperature. To meet the acceptance criteria, the difference between the stability and fresh samples should be within ±15%.

**RESULTS AND DISCUSSION**

The mean absolute recoveries of EES determined at 28.0, 475.0 and 1050.0 ng/mL were 58.1% (RSD 4.1%), 64.7% (RSD 2.3%) and 65.4% (RSD 5.4%), respectively. The mean absolute recovery of imipramine was 68.6% (RSD 2.2%).

Minimal matrix effect for EES was observed from the six different plasma lots tested. The RSD of the area ratios of post spiked recovery samples at LQC and HQC levels were less than 2.7% for EES. For the internal standard the RSD of the area response over both LQC and HQC levels was less than 7.8%. This indicated that the extracts were “clean” with no co-eluting compounds influencing the ionization of the analyte and the internal standard.

The high selectivity of MS-MS detection allowed the development of a very specific and rapid method for the determination of EES in plasma. Representative chromatograms obtained from blank plasma and blank plasma spiked with LLOQ standard is presented Figure 2(a) and Figure 2(b) respectively. No significant interfering peak of endogenous compounds was observed at the retention time of analyte in blank human plasma containing Na Heparin as the anti-coagulant in ten different plasma lots which was compared versus six replicates of extracted samples at the LLOQ level.

The LLOQ, defined as that concentration of erythromycin ethylsuccinate which can still be determined with acceptable precision (%RSD < 20) and accuracy (bias within ±20%) was found to be 10.0 ng/mL. Results of the intra-batch and inter-batch validation assays are presented in Tables 1 and 2, respectively. The inter-batch and intra-batch precision were ≤ 7.0% and ≤ 6.1% whereas the inter-batch and intra-batch accuracy in terms of % bias were within the range of -6.8 to -2.9 and -8.6 to -4.2 for EES respectively.

**Table 1**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Spiked conc. (ng/mL)</th>
<th>Mean calculated conc. (ng/mL)</th>
<th>%RSD</th>
<th>% Bias</th>
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<tr>
<td>Erythromycin ethylsuccinate</td>
<td>10.2</td>
<td>9.7</td>
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<td></td>
<td>28.0</td>
<td>25.6</td>
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<td></td>
<td>475.0</td>
<td>455.0</td>
<td>3.2</td>
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<td></td>
<td>1050.0</td>
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**Table 2**

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<tr>
<th>Analyte</th>
<th>Spiked conc. (ng/mL)</th>
<th>Mean calculated conc. (ng/mL)</th>
<th>%RSD</th>
<th>% Bias</th>
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<tr>
<td></td>
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<td></td>
<td>1050.0</td>
<td>981.6</td>
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Figure 1: Chemical Structure for (a) Erythromycin ethylsuccinate and (b) Imipramine
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Bench top and processed (autosampler) stability for EES were performed at LQC and HQC levels. The results revealed that erythromycin ethylsuccinate was stable in plasma for at least 6 h at room temperature and 50 h in auto sampler at 4 °C. It was confirmed that repeated freeze and thawing (five cycles) of spiked plasma samples at LQC and HQC level did not affect the stability of EES. EES was found stable for minimum five freeze and thaw cycles. The long term stability results also indicated that EES was stable in human plasma for up to 99 days at a storage temperature of -70 °C. This period of long term stability was sufficient enough to cover the entire storage period from first day of storage of the plasma samples to the last day of analysis.

During method development different options were evaluated to optimize sample extraction, detection parameters and chromatography. Best signal for the analyte was achieved with the ESI positive ion mode. In the nonionic forms, the strong binding of analytes to the copolymer of SPE cartridge enables sufficient clean up. A mobile phase containing buffer ammonium acetate and formate salt at different molarity and acetonitrile in varying combinations was tried during the initial development stages. The effect of pH of buffer also checked on sensitivity and peak shape. But the best signal and peak shape for erythromycin ethylsuccinate was achieved using a mobile phase of 0.1% (v/v) formic acid in deionised water in combination with acetonitrile (20:80 v/v). Use of Hypurity Advance (100mm x 4.6mm, 5µm) column resulted in reduced flow rate (0.4 ml/min) and reduced run time. The retention times for EES and imipramine was ~ 1.6 minutes.

Imipramine used as internal as ionization, retention and extraction characteristics of imipramine were found to be similar to that of EES and hence it was selected as the internal standard of choice.

The validated method was employed to analyse plasma samples containing EES obtained from a single oral dose of 250mg/5mL erythromycin (Ethylsuccinate) in 12 healthy volunteers under fasting conditions. Representative chromatogram obtained from real subject sample analysis at concentration of 856 ng /mL EES is also presented in Figure 2(c). The study design was an open label, single-period, single dose, bioavailability study. The study conduced was carried out after approval from an independent ethics committee and obtaining written consent from the volunteers. All the statistical parameters computed using SAS 9.1.3 software post-study are tabulated in Table 3.

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<th>Parameters</th>
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<th>T&lt;sub&gt;max&lt;/sub&gt; (h)</th>
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<th>AUC&lt;sub&gt;0-inf&lt;/sub&gt;</th>
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<td>2005.58</td>
<td>2254.53</td>
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</table>

CONCLUSION

A robust, simple and rapid method for the estimation of erythromycin ethylsuccinate in human plasma was developed and validated, using high-performance liquid chromatographic separation with tandem mass spectrometric detection. The validated method was used in a pharmacokinetic study in which 12 healthy volunteers were enrolled; each subject dosed 250 mg/5mL erythromycin oral suspension (as ethylsuccinate) as a single oral dose. With an LLOQ of 10.0 ng /mL, pharmacokinetic profiles of the drug could be constructed for up to 12 h. The method allows higher sample throughput due to the short chromatography run time (3.0 minutes) and simple sample preparation. Robust LC-MS-MS performance was observed, with acceptable variation in instrument response within batches. This method is an excellent analytical option for rapid quantification of erythromycin ethylsuccinate in human plasma.

References


[5] Pichichero, M. E.; Pichichero, D. M. Diagnosis of Penicillin, Amoxicillin, and Cephalosporin Allergy: Reliability of


