Short communication

Liquid chromatography–tandem mass spectrometric assay for eltrombopag in 50 μL of human plasma: A pharmacokinetic study

Rambabu Maddela a, b, *, Ramakrishna Gajula b, Nageswara Rao Pilli a, b, Sridhar Siddiraju c, Srinubabu Maddela a, Ajitha Makula a, *

a Center for Pharmaceutical Sciences, Jawaharlal Nehru Technological University, Kukatpally, Hyderabad 500 085, India
b Piramal Clinical Research Laboratories, Ramanthapur, Hyderabad 500 013, India
c Department of Pharmaceutical Chemistry, Malla Reddy College of Pharmacy, Maisammaguda, Secunderabad 500010, India

A R T I C L E   I N F O

Article history:
Received 22 February 2014
Received in revised form 10 April 2014
Accepted 26 April 2014
Available online 2 May 2014

Keywords:
Eltrombopag
Human plasma
Protein precipitation (PP)
LC–MS/MS
Pharmacokinetics

A B S T R A C T

Eltrombopag is a thrombopoietin receptor agonist, used in the treatment of thrombocytopenia. This paper describes a liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay method for the determination of eltrombopag in human plasma samples using eltrombopag 13C4 as internal standard (IS). Analyte and the IS were extracted from 50 μL of human plasma using protein precipitation technique with no drying, evaporation and reconstitution steps. The chromatographic separation was achieved on a C18 column by using a mixture of 10 mM ammonium formate (pH 3) and acetonitrile (10:90, v/v) as the mobile phase at a flow rate of 1.0 mL/min. The linearity of the method was established in the concentration range 50.0–1000 ng/mL with r2 ≥ 0.99. The intra-day and inter-day precision and accuracy results in four validation batches across five concentration levels were well within the acceptance limits. The proposed method was found to be applicable to pharmacokinetic studies.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Eltrombopag is an orally bioavailable thrombopoietin receptor agonist that has been approved for the treatment of thrombocytopenia in patients with chronic immune (idiopathic) thrombocytopenic purpura (ITP). The drug used in the patients with insufficient response to corticosteroids, immunoglobulins, or splenectomy. Eltrombopag should be used only in patients with ITP whose degree of thrombocytopenia and clinical condition increases the risk for bleeding [1–4]. The drug has high plasma protein binding affinity and primarily eliminated via metabolism in the liver and gastrointestinal tract [5].

As per the literature, very few analytical methods have been reported for the determination of eltrombopag in biological samples, including liquid chromatography/mass spectrometric methods [6–8] and high performance liquid chromatographic (HPLC) methods [9,2]. Most of the analytical methods reported so far were only for investigation of pharmacokinetics properties of the drug. Quantitative determination of eltrombopag in human plasma remains difficult due to its physicochemical properties and polarity. To address the pharmacokinetics of the new formulations of eltrombopag, a sensitive and specific method that allows accurate determination eltrombopag concentrations in human plasma is needed. The conventional HPLC methods must sacrifice time, resolution or sensitivity. Therefore, it is necessary to develop fast or ultra-fast methods such as LC–MS/MS without any loss of separation efficiency and sensitivity. Thus, the aim of this study was to develop a simple, rapid, and sensitive LC–MS/MS method for determination of eltrombopag in human plasma.

This paper presents for the first time, the development and validation of a simple, selective and sensitive method, which employs protein precipitation (PP) technique for sample preparation and liquid chromatography with electrospray ionization–tandem mass spectrometry for the quantitation of eltrombopag in human plasma. We have also employed eltrombopag stable labeled isotope eltrombopag 13C4 as an internal standard (IS), to avoid the potential matrix effect related problems and variability in recovery between analyte and the IS. The developed method was successfully applied to pharmacokinetic study in healthy male volunteers following oral administration of eltrombopag is described.
2. Experimental

2.1. Standards and reagents

Eltrombopag olamine reference standard (99.69% pure) was obtained from Hetero Drugs Limited (Hyderabad, India). Eltrombopag 13C4 (99.66% pure) was employed as an internal standard and was obtained from CLEARSYNTH LABS Limited (Mumbai, India). HPLC grade acetonitrile and methanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). Analytical grade ammonia solution (25%), dimethyl sulfoxide and ammonium formate were purchased from Merck Ltd. (Mumbai, India). Water used for the LC–MS/MS analysis was prepared by using Milli-Q water purification system procured from Millipore (Bangalore, India). The control human plasma sample was procured from Deccan’s Pathological Lab’s (Hyderabad, India).

2.2. LC–MS/MS instrument and conditions

An HPLC system (Shimadzu, Kyoto, Japan) consisting of a Kromasil 100-5 C18 column 100 mm × 4.6 mm, 5 μm (Make: AkzoNobel, Pune, India), a binary LC-20AD prominence pump, an auto sampler (SIL-HTc) and a solvent degasser (DGU-20A3) was used for the study. Aliquot of 10 μL of the processed samples were injected into the column, which was kept at room temperature (20 ± 5 °C). An isotropic mobile phase consisting of a mixture of 10 mM ammonium formate (pH 3) and acetonitrile (10:90, v/v) was used to separate the analyte from the endogenous components and pumped at a flow rate of 1.0 mL/min into the electrospray ionization chamber of the mass spectrometer. Quantification was achieved with MS–MS detection in negative ion mode for the analyte and the IS using an AB Sciex API-4000 mass spectrometer (Foster City, CA, USA) equipped with a TurboionSpray™ interface at 550 °C. The ion spray voltage was set at −4500 V. The source parameters viz. the nebulizer gas (GS1), auxiliary gas (GS2), curtain gas and collision gas were set at 20, 25, 40, and 8 psi, respectively. The compound parameters viz. the declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were −65, −30, −10, −9 V for eltrombopag and −86, −38, −10, −10 V for the IS. Detection of the ions was carried out in the multiple–reaction monitoring mode (MRM), by monitoring the transition pairs of m/z 441.20 precursor ion to the m/z 201.10 for eltrombopag and m/z 445.20 precursor ion to the m/z 205.10 product ion for the IS. Quadrupoles (Q1 and Q3) were set on unit resolution. Date acquisition was performed with Analyst Software™ (version 1.4.2).

2.3. Standard stock, calibration standards and quality control sample preparation

The standard stock solution of eltrombopag (0.2 mg/mL) was prepared by dissolving accurately weighted amount in a mixture of methanol and 0.2% ammonia solution in water (60:40, v/v, diluent). Working solutions for calibration standards (CCs) and quality control (QCs) samples were prepared by appropriate dilution in the diluent. The primary stock solution of the IS (1 mg/mL) was prepared by dissolving requisite amount of eltrombopag 13C4 in dimethyl sulfoxide (DMSO). A working concentration of the internal standard (30 μg/mL) solution was prepared in the diluent. Standard stock and working solutions used were stored at 2–8 °C for 18 days (data not shown).

CCs were made at 50.0, 100, 250, 500, 1001, 2002, 4003, 8006 and 10007 ng/mL concentrations, respectively, while QCs were prepared at 51.3 ng/mL (lower limit of quantitation, LLOQ), 151 ng/mL (low quality control, LQC), 1207 ng/mL (medium quality control, MQC1), 5106 ng/mL (MQC2) and 8509 ng/mL (high quality control, HQC). The CCs and QCs were stored in the freezer at −70 ± 10 °C until use.

2.4. Sample preparation procedure

All frozen subject samples, calibration standards and quality control samples were thawed and allowed to equilibrate at room temperature prior to analysis. The samples were vortexed to mix for 10 s prior to spiking. A 50 μL aliquot of human plasma sample was mixed with 25 μL of the internal standard working solution (30.0 μg/mL of eltrombopag 13C4). To this, 2 mL of mobile phase was added. After vortex-mixing for 30 s and centrifugation at 4000 rpm for 20 min, the supernatant was transferrered to a prelabelled autosampler loading vials and an aliquot of 10 μL was injected into LC–MS/MS system.

2.5. Method validation procedures

The validation of the above method was carried out as per US FDA guidelines [10]. The parameters determined were selectivity, matrix effect, sensitivity, linearity, precision and accuracy, recovery, dilution integrity and stability. The selectivity of the method was assessed in six different sources of plasma, of which, four were normal K2 EDTA plasma and one each of lipemic and heoloyzed plasma. Sensitivity of the method was assessed by analyzing six sets of spiked plasma samples at lowest level of the calibration curve concentrations (LLOQ). Qualitative ion suppression/enhancement effects (matrix effect) on the MRM LC–MS/MS sensitivity were evaluated by post column infusion technique. A standard solution (aqueous sample) containing eltrombopag (at MQC1 level) was infused post-column into the mobile phase at 10 μL/min using infusion pump. Also, an aliquot of 10 μL of post extraction spiked sample (at MQC1 level) was then infused post–column into the mobile phase at similar flow rate. Matrix effect was also evaluated with IS normalized matrix factor (MF), was assessed by comparing the mean area response of post-extraction spiked samples with mean area of aqueous samples (neat samples) prepared in mobile phase solutions at LQC and HQC levels. IS-normalized MF was calculated using the below formula:

\[
\text{IS normalized matrix factor} = \frac{\text{peak response area ratio in presence of matrix ions}}{\text{mean peak response area ratio in absence of matrix ions}}
\]

The linearity of the method was established by analysis of four calibration curves (CC) containing nine non-zero concentrations. In addition, each curve contains one blank plasma sample and one blank plasma sample with internal standard (zero standard). Each CC was analyzed individually by least square weighted (1/x^2) linear regression. Intra-day accuracy and %RSD were evaluated by replicative analysis of plasma samples (LLOQ QC, LQC, MQC1, MQC2 and HQC) on the same day. Inter-day accuracy and %RSD were assessed by analyzing four batches of samples on three consecutive days. The % RSD at each concentration level from the nominal concentration should not be greater than 15%, except for LLOQ QC where it should be 20%. The accuracy (%) must be within ±15% of their nominal value at each QC level except LLOQ QC where it must be within ±20%.

Recovery for the analyte and the IS was calculated by comparing the mean detector response of six sets of pre-extraction spiked samples (spiked before extraction) to that of six sets of neat samples (aqueous) at each concentration level. Recovery of the analyte was determined at a concentration of 151 (LQC), 5106 (MQC2) and 8509 (HQC) ng/mL whereas for the IS was determined at concentration of 30.0 μg/mL. Dilution integrity was performed to extend the upper concentration limit with acceptable precision and accuracy.
Six replicates each at a concentration of about 1.65 times of the uppermost calibration standard were diluted two- and four-fold with screened blank plasma. The diluted samples were processed and analyzed with un-diluted calibration curve standards.

Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The stock solution stability at room temperature and refrigerated conditions (2–8 °C) was performed by comparing the area response of the analyte (stability samples) with the response of the sample prepared from fresh stock solution. Bench top stability (17 h), processed samples stability (autosampler stability for 48 h, wet extract stability for 42 h and re-injection stability for 41 h), freeze-thaw stability (4 cycles), long-term stability (50 days) were performed at LQC and HQC levels using six replicates at each level. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (±15% SD) and %RSD (≤15%).

2.6. Pharmacokinetic study protocol design

A pharmacokinetic study was performed in healthy male subjects (n = 8). All the volunteers provided with written informed consent and were fasted for 12 h before the drug formulation administration. Blood samples were collected following oral administration of 50 mg eltrombopag tablet at pre-dose and 1, 1.5, 2, 2.33, 2.67, 3, 3.33, 3.67, 4, 4.33, 4.67, 5, 5.5, 6, 6.5, 7, 8, 10, 12, 16, 24, 36, 48, 72, and 96 h and collected in K3 EDTA vacutainer (5 mL) collection tubes (BD, Franklin, NJ, USA). The tubes were centrifuged at 3200 rpm for 10 min and the plasma was collected. The collected plasma samples were stored at -70 ± 10 °C till their use. Plasma samples were spiked with the IS and processed as per the extraction procedure described earlier. The main pharmacokinetic parameters of eltrombopag were calculated by non-compartmental model using WinNonlin Version 5.2. As per FDA recommendations it is necessary to perform of incurred sample reanalysis (ISR) using spiked subject samples. ISR is to verify the reliability and reproducibility of the reported subject sample analyte concentrations. Hence an ISR was performed by selecting the 12 subject samples (2 samples from each subject) near Cmax and the elimination phase in the pharmacokinetic profile of the drug. The ISR values were compared with the initial values. The percent change in the value should not be more than ±20% [11,12].

3. Results and discussion

3.1. Method development

Mass parameters were optimized by infusing the standard analyte solution of 100 ng/mL into the mass spectrometer using electrospray as the ionization source and operating in the MRM mode. The signal intensities obtained in negative mode were much higher than those in positive mode. Deprotonated form of analyte and the IS, [M – H]⁻ ion was the parent ion in the Q1 spectrum and was used as the precursor ion to obtain Q3 product ion spectra. The most sensitive mass transition was observed from m/z 441.20 to 201.10 for eltrombopag and from m/z 445.20 to 205.10 for the IS. The dwell time for each transition was set at 200 ms.

Method development includes the mobile phase selection, flow rate, column type and injection volume. Different combinations of methanol/acetonitrile with acidic buffers (ammonium acetate/ammonium formate-acetic acid/formic acid) in different volume ratios were tested. It was observed that 10 mM ammonium formate (pH 3) and acetonitrile (10:90, v/v) as the mobile phase was most appropriate to give best sensitivity, efficiency and peak shape. Kromasil 100-5C18 (100 mm × 4.6 mm, 5 µm) column gave good peak shape and response even at lowest concentration level for the analyte and the IS. The mobile phase was operated at a flow rate of 1.0 mL/min. The retention time of analyte and the IS were low enough (2.50 and 2.50 min) allowing a run time of 3.50 min.

A simple protein precipitation (PP) technique was employed for the sample preparation in this work and provides high recoveries of the analyte and the IS. Also, eltrombopag had more protein binding nature and were precipitated easily with the mobile phase. Precipitation with mobile phase caused the lowest matrix effect with better peak shape compared to other organic solvents (methanol and acetonitrile). The use of stable labeled isotopes of the analyte as an IS is recommended for bioanalytical assays to increase assay precision and limit variable recovery between analyte and the IS. At the initial stages of this work, several compounds were investigated to find a suitable IS and finally eltrombopag stable labeled isotope eltrombopag 13C4 was found to be best for the present purpose.

3.2. Selectivity and chromatography

Sample preparation by PP afforded clean chromatograms with no interfering peaks as evident from representative MRM ion chromatograms of extracted blank plasma sample (without analyte and the IS), blank plasma sample spiked with the IS, eltrombopag at LLOQ and an actual plasma sample at 4.0 h (Fig. 1).

3.3. Matrix effect

The chromatograms for post column infusion experiment show the absence of signal suppression or enhancement at the retention time of eltrombopag and the IS (Fig. 2). Also, the average matrix factor valve calculate as the response of the post spiked sample/response of neat sample at LQC and HQC level was 0.99 and 1.01, which indicated negligible suppression or enhancement.

3.4. Linearity, sensitivity, precision and accuracy

Four eltrombopag calibration curves were linear over the concentration range of 50.0–1000 ng/mL with a correlation coefficient (r²) ≥ 0.9970. The mean linear equation obtained for eltrombopag was y = (0.000093 ± 0.000004)x + (0.001240 ± 0.000979), where y is the peak area ratio of the analyte/IS and x the concentration of the analyte. The accuracy and %RSD for the calibration standards ranged from 92.8% to 106% and 0.38% to 11.68%.

The lowest limit of reliable quantification for the analyte was set at the concentration of the LLOQ (50.0 ng/mL). The %RSD and accuracy at LLOQ concentration were found to be 3.04% and 105%, respectively. The results for intra-day and inter-day %RSD and accuracy in plasma quality control samples are summarized in

Table 1

<table>
<thead>
<tr>
<th>Quality control</th>
<th>Run</th>
<th>Concentration found</th>
<th>%RSD</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLOQ</td>
<td>52±1.71</td>
<td>3.28</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>LQC</td>
<td>148±2.67</td>
<td>1.81</td>
<td>97.8</td>
<td></td>
</tr>
<tr>
<td>MQC1</td>
<td>1277±11.3</td>
<td>0.92</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>MQC2</td>
<td>4958±34.6</td>
<td>0.70</td>
<td>97.1</td>
<td></td>
</tr>
<tr>
<td>HQC</td>
<td>8376±80.5</td>
<td>0.96</td>
<td>98.4</td>
<td></td>
</tr>
<tr>
<td>Inter-day variations (24 replicates at each concentration)</td>
<td>51.5±3.26</td>
<td>6.33</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>LQC</td>
<td>148±2.39</td>
<td>1.61</td>
<td>98.3</td>
<td></td>
</tr>
<tr>
<td>MQC1</td>
<td>1245±28.7</td>
<td>2.31</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td>MQC2</td>
<td>5016±91.1</td>
<td>1.82</td>
<td>98.2</td>
<td></td>
</tr>
<tr>
<td>HQC</td>
<td>8562±291</td>
<td>3.39</td>
<td>101</td>
<td></td>
</tr>
</tbody>
</table>

Spiked concentrations of LLOQ, LQC, MQC1, MQC2 and HQC are 51.3, 151, 1207, 5106 and 8509 ng/mL, respectively.
Table 1. The intra-batch %RSD ranged from 0.70 to 3.28% and the accuracy was within 97.1–102%. For inter-batch experiment, the %RSD varied from 1.61 to 6.33% and the accuracy was within 98.2–103%.

3.5. Recovery and dilution integrity

The mean overall recovery of eltrombopag was 73.5 ± 1.86% with the %RSD range of 1.25–2.99% and the recovery of the IS was
75.5%. The recoveries of analyte and the IS were good and reproducible.

The upper concentration limit of eltrombopag can be extended to 16531 ng/mL by using two- and four-fold dilution with screened human blank plasma. The %RSD for dilution integrity of half and quarter dilution was found to be 0.90% and 1.45%, while the accuracy results were found to be 103% and 97.9%, respectively.

3.6. Stability studies

In various stability experiments carried out namely bench top stability (17 h), autosampler stability (48 h), repeated freeze-thaw cycles (4 cycles), reinjection stability (41 h), wet extract stability (42 h at 2–8 °C) and long-term stability at −70 °C for 50 days the mean % nominal values of the analyte were found to be within ±15% of the predicted concentrations for the analyte at their LQC and HQC levels (Table 2). Therefore, the results were found to be within the acceptable limits during the entire validation.

3.7. Pharmacokinetic study and incurred samples reanalysis

The proposed method was successfully used to quantify eltrombopag plasma concentration for a pharmacokinetic study in healthy adult male subjects (n = 8) under fasting condition. Fig. 3 depicts the mean plasma concentration vs time profile of eltrombopag in health subjects. The maximum concentration (C\textsubscript{max}) in plasma (6243 ± 965 ng/mL) for eltrombopag was attained at 3.96 ± 0.63 h (t\textsubscript{max}). The area under the plasma concentration time curve from time zero to last measurable time point (AUC\textsubscript{0-\infty}) and area under the plasma concentration time curve from time zero to infinity

Table 2

<table>
<thead>
<tr>
<th>Stability test</th>
<th>QC (spiked concentration) (ng/mL)</th>
<th>Concentration found (ng/mL)</th>
<th>%RSD</th>
<th>Accuracy/ stability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Process\textsuperscript{a}</td>
<td>151</td>
<td>164 ± 3.31</td>
<td>2.02</td>
<td>108</td>
</tr>
<tr>
<td>Process\textsuperscript{b}</td>
<td>8509</td>
<td>8550 ± 155</td>
<td>1.81</td>
<td>100</td>
</tr>
<tr>
<td>Bench top\textsuperscript{c}</td>
<td>151</td>
<td>162 ± 4.21</td>
<td>2.59</td>
<td>108</td>
</tr>
<tr>
<td>Bench top\textsuperscript{d}</td>
<td>8509</td>
<td>8574 ± 66.7</td>
<td>0.78</td>
<td>101</td>
</tr>
<tr>
<td>FT\textsuperscript{e}</td>
<td>151</td>
<td>163 ± 4.18</td>
<td>2.57</td>
<td>108</td>
</tr>
<tr>
<td>FT\textsuperscript{f}</td>
<td>8509</td>
<td>8750 ± 107</td>
<td>1.23</td>
<td>103</td>
</tr>
<tr>
<td>Re-injection\textsuperscript{g}</td>
<td>151</td>
<td>154 ± 1.96</td>
<td>1.28</td>
<td>102</td>
</tr>
<tr>
<td>Re-injection\textsuperscript{h}</td>
<td>8509</td>
<td>8452 ± 75.8</td>
<td>0.90</td>
<td>99.3</td>
</tr>
<tr>
<td>Long-term\textsuperscript{i}</td>
<td>151</td>
<td>157 ± 2.69</td>
<td>1.71</td>
<td>104</td>
</tr>
<tr>
<td>Long-term\textsuperscript{j}</td>
<td>8509</td>
<td>8409 ± 105</td>
<td>1.25</td>
<td>98.8</td>
</tr>
</tbody>
</table>

\textsuperscript{a} After 48 h in autosampler at 15 °C.
\textsuperscript{b} After 42 h at room temperature.
\textsuperscript{c} After 17 h at room temperature.
\textsuperscript{d} After 4 freeze and thaw cycles.
\textsuperscript{e} After 41 h of reinjection.
\textsuperscript{f} At −70 °C for 50 days.

Fig. 2. Post-column analyte infusion MRM LC–MS/MS chromatogram for eltrombopag at MQC1 level (A) aqueous sample and (B) post extraction spiked sample.

Fig. 3. Mean plasma concentration-time profile of eltrombopag in human plasma following oral dosing of eltrombopag (50 mg tablet) to healthy volunteers (n = 8).
time point (AUC$_{0-\text{inf}}$) for eltrombopag were 76086 ± 14747 and 83468 ± 17070 ng × h/mL, respectively. The terminal half-life ($t_{1/2}$) was found to be 36.7 ± 9.31 h. The differences in concentrations between the ISR and the initial values for all the tested samples were less than 15%, indicating good reproducibility of the present method.

4. Conclusions

The proposed LC–MS/MS method is simple, rapid, specific and sensitive for determination of eltrombopag in human plasma and is fully validated according to commonly acceptable FDA guidelines. The current method has shown acceptable precision and adequate sensitivity for the quantification of eltrombopag in human plasma samples obtained for pharmacokinetic studies. The method employs very low plasma volume (50 μL) for processing by a simple and one step PP technique. The method provided good linearity. From the results of all the validation parameters, we can conclude that the developed method can be useful for bioavailability and bioequivalence (BA/BE) studies and routine therapeutic drug monitoring with the desired precision and accuracy.

Acknowledgment

The authors gratefully acknowledge Piramal Clinical Research Laboratories (Hyderabad, India) for providing necessary facilities to carry out this work.

References