

# Sensitive Liquid Chromatography Positive Electropray Tandem Mass Spectrometry Method for the Quantitation of Tegaserod in Human Plasma Using Liquid–Liquid Extraction

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## Abstract

A sensitive and rapid high-performance liquid chromatography–positive ion electropray tandem mass spectrometry method is developed and validated for the quantitation of tegaserod in human plasma. Following liquid–liquid extraction, the analytes are separated using an isocratic mobile phase on a reversed-phase column and analyzed by tandem mass spectrometry in the multiple reaction monitoring mode using the respective (M+H)<sup>+</sup> ions,  $m/z$  302 to 173 for tegaserod and  $m/z$  409 to 228 for the internal standard. The assay exhibits a linear dynamic range of 100–10000 pg/mL for tegaserod in human plasma. The lower limit of quantitation is 100 pg/mL with a relative standard deviation of less than 7%. Acceptable precision and accuracy are obtained for concentrations over the standard curve range. A run time of 2.0 min for each sample makes it possible to analyze more than 250 human plasma samples per day. The validated method is successfully used to analyze human plasma samples for application in pharmacokinetic, bioavailability, or bioequivalence studies.

## Introduction

Tegaserod is a serotonin receptor agonist, which, unlike any other agent used for irritable bowel syndrome (IBS), is a selective and partial agonist at the 5-hydroxytryptamine receptor subtype 4 (5-HT<sub>4</sub>) (1,2). Tegaserod was approved by the United States Food and Drug Administration for the short-term treatment of constipation-predominant IBS in women and for the treatment of chronic idiopathic constipation in patients younger than 65 years (3). In clinical trials, tegaserod had favorable effects on gastrointestinal transit time and was safe and effective in treating symptoms associated with constipation-predominant IBS in women.

The bioanalytical component of a pharmacokinetic study requires a drug assay with simplicity, sensitivity, selectivity, small volume requirements, and rapid turnaround time. Very few methods for the quantitation of tegaserod in plasma have been reported. Three different analytical methods, namely high-performance liquid chromatography (HPLC)–UV detection (4), gas chromatography–mass spectrometry (GC–MS) (5), and liquid chromatography–tandem mass spectrometry (LC–MS–MS) (6,7) have been reported for routine determination of tegaserod concentrations in plasma. The method of Sonu et al. (6) involves solid-phase extraction of tegaserod from plasma, using the Waters Oasis HLB 1 cm<sup>3</sup> (30 mg) cartridges. The sensitivity of the method was low (200 pg/mL) and the chromatographic run time was longer (4 min). Recently, Zou et al. (7) reported an LC–MS–MS method with a lower limit of quantitation (LLOQ) of 50 pg/mL by liquid–liquid extraction. Though the method is sensitive, plasma consumption was very high (1 mL), and chromatographic run time was long (7 min).

The purpose of the present investigation was to explore the high selectivity and sensitivity of a triple-quadrupole MS system operated in MS–MS mode with an electropray interface for the development and validation of a rapid LC–MS–MS method in multiple reaction monitoring (MRM) mode for the quantitation of tegaserod in human plasma. It was essential to establish a method capable of quantifying tegaserod at concentrations down to 100 pg/mL using low volumes of plasma. Generally, any method should be able to detect drug concentrations up to 3 half-lives of drug. In other words, a method should have a sensitivity limit of at least 10 times lower than peak plasma concentrations ( $C_{\max}$ ). With lower dosages of tegaserod (4 or 6 mg), the average  $C_{\max}$  concentrations are between 1000 to 2000 pg/mL (7). Therefore, the current method was developed to have a sensitivity limit of 100 pg/mL. At the same time, it was expected that this method would be efficient in analyzing a large number of plasma samples obtained for pharmacokinetic, bioavailability, or bioequivalence studies after therapeutic doses of tegaserod.

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## Experimental

### Chemicals

Tegaserod hydrochloride monohydrate drug substance was obtained from Torrent Research Centre (Ahmedabad, India), and tamsulosin hydrochloride (internal standard; IS) was obtained from Suven Life Sciences Ltd. (Hyderabad, India). Chemical structures are presented in Figure 1. HPLC-grade LiChrosolv acetonitrile and HPLC-grade LiChrosolv methanol were purchased from Merck (Darmstadt, Germany). Formic acid was purchased from Merck (Worli, Mumbai, India). HPLC-grade water from Milli-Q system (Millipore, Bedford, MA) was used. All other chemicals were of analytical grade.

### LC-MS-MS instrument and conditions

The HPLC SIL HTC system (Shimadzu Corporation, Kyoto, Japan) is equipped with LC-AD VP binary pump, a DGU20A5 degasser, and a SIL-HTC auto sampler equipped with a CTO-10AS VP thermostatted column oven. The chromatography was performed using Chromolith Speed ROD RP-18e, 100 × 4.6 mm at 30°C temperature. The isocratic mobile phase composition was a mixture of 0.03% formic acid–acetonitrile (25:75, v/v), which was pumped at a flow-rate of 1.5 mL/min with split ratio of load to waste 10:90.

Mass spectrometric detection was performed on an API 3000 triple quadrupole instrument (MDS-SCIEX, Concord, Ontario, Canada) using MRM. A turboionspray interface operating in positive ionization mode was used. Typical source conditions were as follows: the turbo-gas temperature was set at 250°C, and the ion spray needle voltage was adjusted at 5500 V. The mass spectrometer was operated at unit resolution for both Q1 and Q3 in the MRM mode, with a dwell time of 200 ms per MRM channel. The precursor/product ion pairs monitored were  $m/z$  302 to 173 for tegaserod and  $m/z$  409 to 228 for the IS. Nebulizer gas was set at 12; curtain gas was at 15 (arbitrary units); collision gas at 6 (arbitrary units). The collision energy was set at 30 for tegaserod and IS, respectively. Data acquisition was performed with analyst 1.4 software (MDS-SCIEX).

### Sample preparation

Standard stock solutions of tegaserod (1 mg/mL) and the IS (1 mg/mL) were separately prepared in methanol. Working solutions for calibration and controls were prepared by appropriate dilution in water–methanol (50:50, v/v; diluent). The IS working solution (80 ng/mL) was prepared by diluting its stock solution with diluent. Working solutions (0.2 mL) were added to drug-free human plasma (9.8 mL) in bulk, to obtain tegaserod concentration levels of 100, 200, 500, 1000, 2000, 3000, 5000, and 10000 pg/mL as a single batch at each concentration. Quality control (QC) samples were also prepared in bulk on an independent weighing of standard drug, at concentrations of 100 (LLOQ), 300 (low), 4000 (medium), and 8000 pg/mL (high) as a single batch at each concentration. The calibration and control bulk samples were divided into aliquots in micro centrifuge tubes (Tarson, 2 mL) and stored in the freezer at below –50°C until analysis.

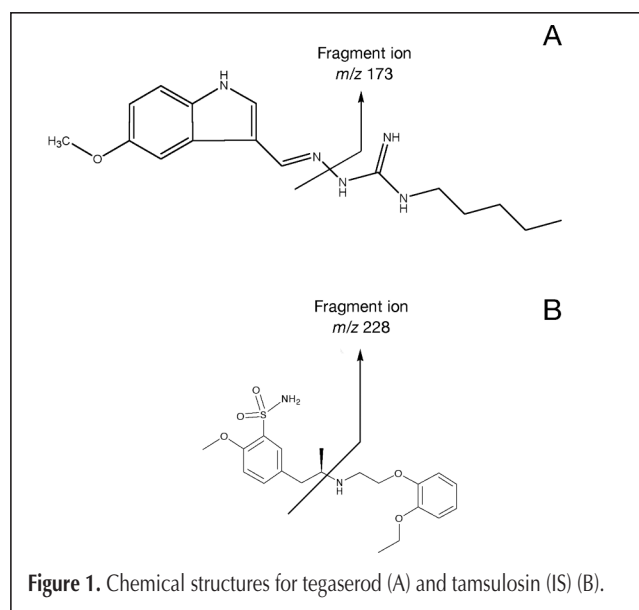
A plasma sample (0.5 mL) was pipetted into a 15-mL glass tube, and then 20  $\mu$ L of IS working solution (80 ng/mL) was added. After vortex-mixing for 10 s, 5 mL aliquot of the extraction solvent, tert-butylmethyl ether, was added and the sample was vortex-mixed for 4 min. The organic layer (4 mL) was transferred to a glass tube and evaporated to dryness using an evaporator at 40°C under a stream of nitrogen. Then the dried extract was reconstituted in 250  $\mu$ L of reconstitution solvent (mobile phase), and a 10- $\mu$ L aliquot was injected into the chromatographic system.

### Bioanalytical method validation

A calibration curve was constructed from a blank sample (a plasma sample processed without the IS), a zero sample (a plasma processed with the IS), and eight non-zero samples covering the total range 100–10000 pg/mL, including the LLOQ. The calibration curves were generated using the analyte to IS peak area ratios by weighted ( $1/x$ ) least-squares linear regression on consecutive days. The acceptance criterion for a calibration curve was a correlation coefficient ( $r$ ) of 0.99 or better, and each back-calculated standard concentration must be within 15% deviation from the nominal value except at the LLOQ, for which the maximum acceptable deviation was set at 20%. At least 67% of non-zero standards were required to meet the previous criteria, including acceptable LLOQ and upper limit of quantitation.

The within-batch precision and accuracy were determined by analyzing four sets of QC samples (LLOQ, low, medium, and high concentrations) each comprised of five replicates in a batch. The between-batch precision and accuracy were determined by analyzing three different batches. The acceptance criteria for within- and between-batch precision were 20% or better for LLOQ and 15% or better for the other concentrations, and the accuracy were 100 ± 20% or better for LLOQ and 100 ± 15% or better for the other concentrations.

Recovery of tegaserod from the extraction procedure was determined by a comparison of the peak area of tegaserod in spiked plasma samples (six each of low, medium, and high QCs) with the peak area of tegaserod in samples prepared by spiking extracted drug-free plasma samples with the same amounts of tegaserod at the step immediately prior to chro-



matography. Similarly, recovery of IS was determined by comparing the mean peak areas of extracted QC samples ( $n = 6$ ) to mean peak areas of IS in samples prepared by spiking extracted drug-free plasma samples with the same amounts of IS at the step immediately prior to chromatography.

The stability of the analyte and IS in human plasma under different temperature and timing conditions, as well as their stability in the stock solutions, were evaluated. QC samples were subjected to short-term room temperature conditions, to long-term storage conditions ( $-50^{\circ}\text{C}$ ), and to freeze-thaw stability studies. All stability studies were conducted at two concentration levels (300 and 8000  $\text{pg/mL}$  as low and high values) with six replicates for each.

## Results and Discussion

### Mass spectrometry

In order to develop a method with the desired LLOQ (100  $\text{pg/mL}$ ), it was necessary to use MS–MS detection, as MS–MS methods provide improved limit of detection and selectivity for trace-mixture analysis. The inherent selectivity of MS–MS detection was also expected to be beneficial in developing a selective and sensitive method.  $(\text{M}+\text{H})^+$  was the predominant ion in the Q1 spectrum and was used as the precursor ion to obtain product ion spectra. The product ion mass spectra, and their proposed rationalizations in terms of fragmentation patterns of tegaserod and IS are illustrated in Figure 2. The most sensitive mass transition was from  $m/z$  302 to 173 for tegaserod and  $m/z$  409 to 228 for the IS.

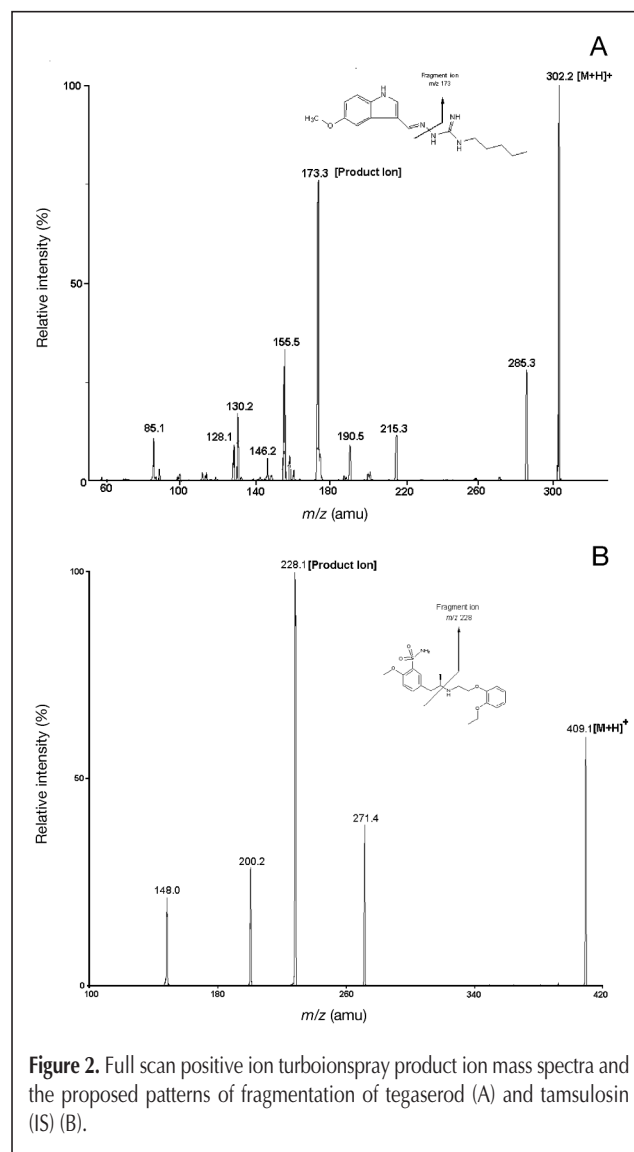
### Method development

Liquid–liquid extraction (LLE) was used for the sample preparation in this work. LLE can be helpful in producing a spectroscopically clean sample and avoiding the introduction of non-volatile materials onto the column and MS system. Clean samples are essential for minimizing ion suppression and matrix effect in LC–MS–MS analyses. *Tert*-butylmethyl ether was found to be optimal, as it can produce a clean chromatogram for a blank plasma sample. The average absolute recoveries of tegaserod from spiked plasma samples was  $63.0 \pm 3.5$ , and the recovery of the IS was  $80.7 \pm 4.6\%$  at the concentration used in the assay (80  $\text{ng/mL}$ ). Recoveries of the analytes and IS were good, and it was consistent, precise, and reproducible. The assay has proved to be robust in high throughput bioanalysis.

The pH of the aqueous phase of the liquid chromatographic mobile phase influences both the chromatographic elution of the compounds and the formation of the  $(\text{M}+\text{H})^+$  molecular ions and is strongly related to their degree of ionization. The  $\text{pK}_a$  values of the analyte and IS were calculated using the MarvinSketch/Swing 4.0.3 software. As both tegaserod and tamsulosin are basic compounds with  $\text{pK}_a$  values 8.7 and 9.78, respectively, the use of slightly acidic solutions favors ionization of the analytes by protonation of their basic sites. Therefore, it was found that positive ionization of the compounds in the electrospray in source increases in acidic mobile phases.

The chromatographic conditions, especially the composition of mobile phase, were optimized through several attempts to achieve good resolution and symmetric peak shapes for the analyte and IS, as well as a short run time. It was found that a mixture of 0.03% formic acid buffer–acetonitrile (25:75, v/v) could achieve this purpose and was finally adopted as the mobile phase. The high proportion of organic solvent eluted the analyte and the IS at retention times of 0.98 and 0.95 min, respectively. A flow rate of 1.5  $\text{mL/min}$  produced good peak shapes and permitted a run time of 2.0 min.

Choosing the appropriate IS is an important aspect of achieving acceptable method performance, especially with LC–MS–MS, where matrix effects can lead to poor analytical results. Ideally, isotopically labeled IS for the analyte should be used, but it is not commercially available. Several compounds were investigated to find a suitable IS, and finally tamsulosin was found to be suitable. Clean chromatograms were obtained and no significant direct interferences in the MRM channels at the relevant retention times were observed. However, in electrospray ionization, signal suppression or enhancement may occur due to co-eluting endogenous components from the



**Figure 2.** Full scan positive ion turboionspray product ion mass spectra and the proposed patterns of fragmentation of tegaserod (A) and tamsulosin (IS) (B).

sample matrix. The importance of including the evaluation of matrix effect in any LC–MS–MS method is outlined in an excellent paper by Matuszewski and co-workers (8). Their data strongly emphasize the need to use a blank matrix from (at least five) different sources/individuals instead of using one blank matrix pool to determine method precision and accuracy. Therefore, all validation experiments in this assay were performed with matrixes obtained from different individuals. In addition, validation experiments were performed using haemolytic and strongly lipemic matrixes. As all data fall within the guidelines, we conclude that the degree of matrix effect was sufficiently low to produce acceptable data, and the method can be considered as valid.

#### Assay performance and validation

The eight-point calibration curve was linear over the concentration range 100–10000 pg/mL. The calibration model was selected based on the analysis of the data by linear regression with/without intercepts and weighting factors ( $1/x$ ,  $1/x^2$ , and none). The best linear fit and least-squares residuals for the calibration curve were achieved with a  $1/x$  weighting factor, giving a mean linear regression equation for the calibration curve of:

$$y = 0.0002 (\pm 2.2359) x + 0.0159 (\pm 2.2288)$$

where  $y$  was the peak area ratio of the analyte to the IS and  $x$  was the concentration of the analyte. The mean correlation coefficient of the weighted calibration curve generated during the validation was  $0.9984 \pm 1.7894$ .

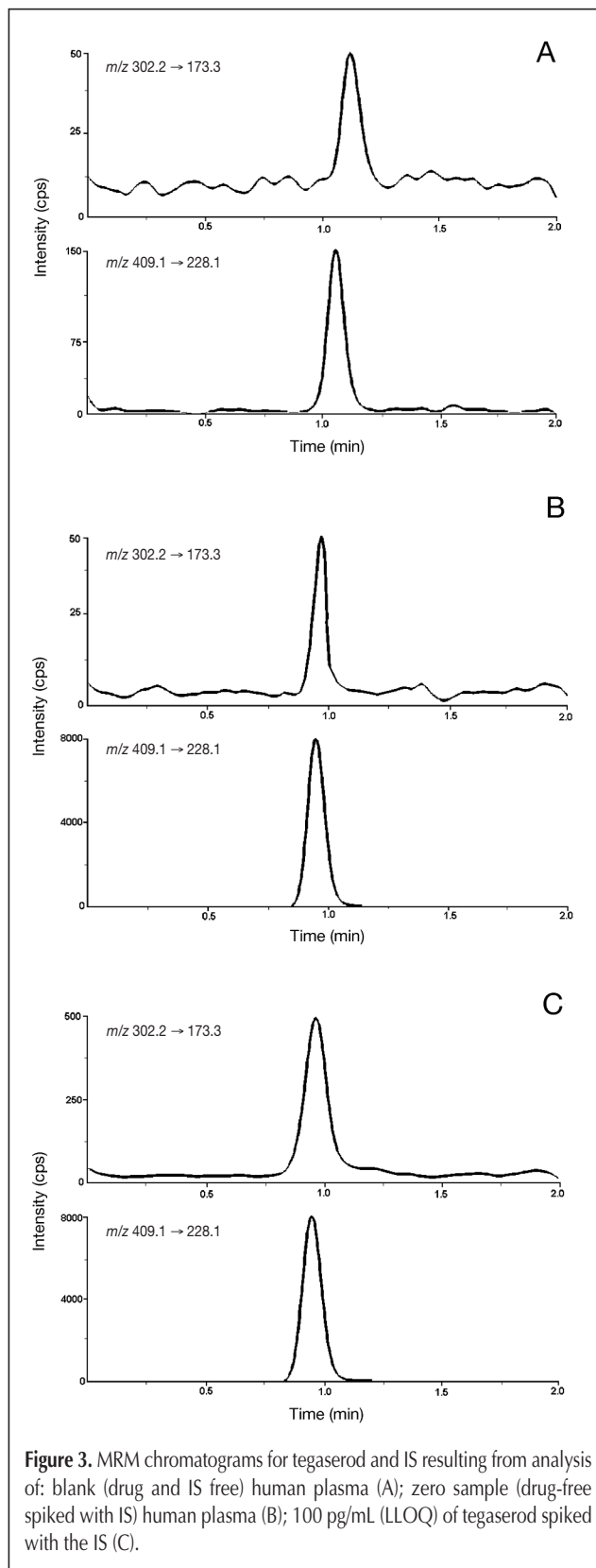
The selectivity of the method was examined by analyzing ( $n = 6$ ) blank human plasma extract (Figure 3A) and an extract spiked only with the IS (Figure 3B). As shown in Figure 3A, no significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free human plasma at the retention time of the analyte. Similarly, Figure 3B shows the absence of direct interference from the IS to the MRM channel of the analyte. Figure 3C depicts a representative ion-chromatogram for the LLOQ (100 pg/mL). Excellent sensitivity was observed for a 10- $\mu$ L injection volume; the LLOQ corresponds to ca. 1 pg on-column.

The LLOQ was defined as the lowest concentration in the standard curve that can be measured with acceptable accuracy and precision, and was found to be 100 pg/mL in human plasma. The mean response for the analyte peak at the assay sensitivity limit (100 pg/mL) was ~10-fold greater than the mean response for the peak in eight blank human plasma samples at the retention time of the analyte. The between-batch precision at the LLOQ was 5.8%, and the between-batch accuracy was 102.1% (Table I). The within-batch precision was 6.9% and the accuracy was 101.8% for tegaserod.

The lower and upper quantitation levels of tegaserod ranged from 100 to 10000 pg/mL in human plasma. For the between-batch experiments the precision ranged from 3.3 to 5.8% and the accuracy from 96.7 to 103.3% (Table I). For the within-batch experiments the precision and accuracy for the analyte met the acceptance criteria ( $< \pm 15\%$ ).

#### Stability studies

For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time exceeding that expected to be encountered during routine sample preparation (~24 h). Samples were



**Figure 3.** MRM chromatograms for tegaserod and IS resulting from analysis of: blank (drug and IS free) human plasma (A); zero sample (drug-free spiked with IS) human plasma (B); 100 pg/mL (LLOQ) of tegaserod spiked with the IS (C).



**Table I. Precision and Accuracy of the Method for Determining Tegaserod Concentrations in Plasma Samples**

Concentration added (pg/mL)	Within-batch (n = 5)			Between-batch (n = 3)		
	Concentration found (mean ± S.D.) (pg/mL)	Precision (%)	Accuracy (%)	Concentration found (mean ± S.D.) (pg/mL)	Precision (%)	Accuracy (%)
100	101.8 ± 7.0	6.9	101.8	101.8 ± 5.9	5.8	102.1
300	310.2 ± 16.2	5.2	103.4	310.2 ± 10.2	3.3	103.3
4000	3994.9 ± 220.3	5.5	99.8	3920.9 ± 151.9	3.8	99.3
8000	7702.2 ± 406.5	5.2	96.2	7702 ± 261.7	3.3	96.7

**Table II. Stability of Tegaserod in Human Plasma**

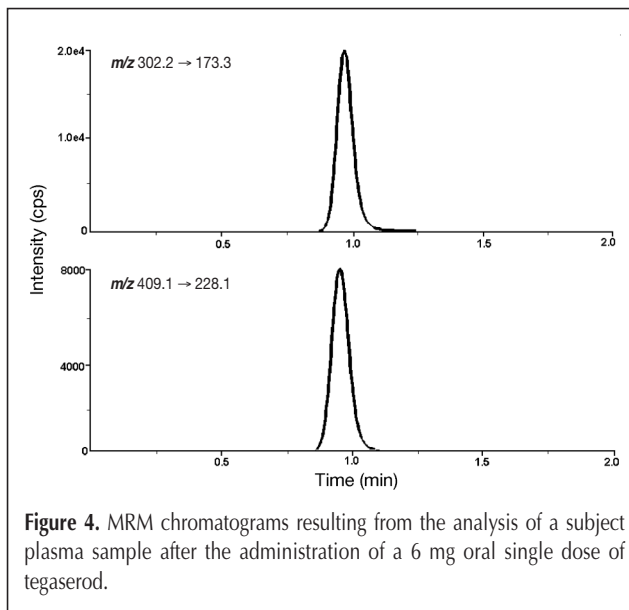
Sample concentration (pg/mL; n = 6)	Concentration found (mean ± S.D.; pg/mL)	Precision (%)	Accuracy (%)
<i>Short-term stability for 24 h in plasma</i>			
300	312	3.2	104.0
8000	7942	2.8	99.3
<i>Three freeze / thaw cycles</i>			
300	304	4.1	101.3
8000	7709	3.7	96.4
<i>Autosampler stability for 20 h</i>			
300	296	2.5	98.7
8000	7875	1.9	98.4
<i>Stability for 30 days at below -50°C</i>			
300	292	5.3	97.3
8000	7718	6.7	96.5

extracted and analyzed as described previously and the results indicate reliable stability behavior under the experimental conditions of the regular analytical procedure (Table II). The stability of QC samples kept in the autosampler for 20 h was also assessed. The results indicate that solutions of the analyte and the IS can remain in the autosampler for at least 20 h without showing significant loss in the quantified values, indicating that samples should be processed within this period of time.

The stability data of the analyte in plasma over three freeze-thaw cycles indicate that the analyte is stable in human plasma for three freeze-thaw cycles, when stored at below -50°C and thawed to room temperature (Table II).

The long-term stability data of the analyte in human plasma stored for a period of 30 days at below -50°C showed reliable stability behavior, as the mean of the results of the tested samples were within the acceptance criteria of ± 15% of the initial values of the controls. These findings indicate that storage of the analyte in plasma samples at below -50°C is adequate, and no stability-related problems would be expected during routine analyses for pharmacokinetic, bioavailability, or bioequivalence studies.

The stability of the stock solutions was tested and established at room temperature for 4 h, 21 h, and under refrigeration (~4°C) for 30 days (data not shown). The results revealed



**Figure 4.** MRM chromatograms resulting from the analysis of a subject plasma sample after the administration of a 6 mg oral single dose of tegaserod.

optimum stability for the prepared stock solutions throughout the period intended for their daily use.

### Application

The method was applied to determine the plasma concentration of tegaserod following a single 6 mg oral administration to 12 healthy subjects. The MRM chromatograms obtained for an extracted plasma sample of a healthy subject who participated in a bioequivalence study are depicted in Figure 4.

### Conclusion

In summary, a method is described for the quantification of tegaserod in human plasma by LC-MS-MS in positive electrospray ionization mode using multiple reaction monitoring and fully validated according to commonly accepted criteria. The current method has shown acceptable precision and adequate sensitivity for the quantitation of tegaserod in human plasma samples obtained for pharmacokinetic, bioavailability, or bioequivalence studies. The desired sensitivity of tegaserod was achieved with an LLOQ of 100 pg/mL, which has a within- and between-batch coefficient of variation of 6.9% and 5.8%, respectively. Many variables related to the electrospray repro-

ducibility were optimized for both precision and sensitivity to obtain these results. The simplicity of the assay and the use of rapid LLE and sample turnover rate of 2.0 min per sample make it an attractive procedure in high-throughput bioanalysis of tegaserod. The method was successfully applied to quantitate the concentrations of tegaserod in a clinical pharmacokinetic study.

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