

## RESEARCH ARTICLE

# LC-MS/MS Bioanalytical Procedure for Atrasentan in Human Plasma: Method Development and Validation

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## ABSTRACT

**Objectives:** The objective of this work is to establish a bioanalytical technique for the quantification of atrasentan. The employed methodology for the detection and quantification of atrasentan in human plasma has a high degree of specificity, sensitivity, and simplicity.

**Methods:** Shimadzu pumps were utilized in conjunction with an Autosampler Agilent Zorbax XDB C18 2.1 × 50 mm, 5 μm column employed as a stationary phase to achieve the chromatographic separation. Acetonitrile (0.1% formic acid) and 5 mM ammonium formate made up the mobile phase, which was optimized to have a 70:30% v/v ratio. Isocratic elution was employed in the experiment, with a flow rate of 0.150 mL/min. The entire cycle took 3.0 minutes, using a 10 μL injection volume.

**Results:** The detection procedure is executed utilizing positive ion mode electrospray ionization (ESI) mass spectrometry under atmospheric pressure. For atrasentan, the precursor to product ion transitions used for quantification was m/z 511.623 to 354.15 and for the internal standard, verapamil, m/z 455.22 to 165.02. The retention times for atrasentan and verapamil were found to be 1.68 and 0.96 minutes, respectively.

**Conclusion:** In accordance with the recommendations of the United States Food and Drug Administration (USFDA) and EMA, it was ascertained that the remaining validation parameters remained within the specified range.

**Keywords:** Human plasma, LC-MS/MS, Atrasentan, Method development, Validation.

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**Conflict of interest:** None

## INTRODUCTION

To delay the spread of prostate cancer that has already spread to other parts of the body, doctors use the oral medicine atrasentan as a monotherapy. It does this by inhibiting the growth-promoting autocrine endothelin protein receptor on cancer cells.<sup>1,2</sup> Figure 1 displays the chemical structure.

Endothelins are signaling molecules that cells create to interact with neighboring cells. They are classified as autocrine/paracrine factors.<sup>3</sup> These signals cause vasoconstriction, cell proliferation, hormone synthesis, angiogenesis, and discomfort. Atrasentan functions as an antagonist by impeding the re-translocation of signals within the cell.<sup>4</sup> Inhibiting endothelin-1, which is overexpressed in prostate cancer cells, impedes the progression of the tumor and alleviates associated distress. Endothelin-1 is found in various types of malignancies, including colorectal, ovarian, breast, cervical, glioma, and pancreatic cancers. It is important to note that atrasentan has the ability to delay the progression of bone lesions. The time to advancement was delayed in all bone

builders.<sup>5,6</sup> While more precise data will not be accessible until the completion of phase 3 trials, results from phases 1 and 2 suggest that this medication may reduce or stabilize the development of PCa in at least two-thirds of users for a minimum of 6 months. There have been no reports of dose-limiting toxicity. This drug appears to be compatible with current therapy.<sup>7,8</sup>

This article provides a detailed description of the outcomes of our liquid chromatography-tandem mass spectrometry (LC-MS/MS) investigation on the quantification of atrasentan in human plasma. Although monitoring plasma concentrations is not a common use in phase III research, it is an example of how analytical work might support investigations on novel drug applications. This technique replaces an earlier high-performance liquid chromatography (HPLC) approach that made use of fluorescence detection. It has been used to provide help for complete phase 3 investigations, with a success rate over 90%.<sup>9-11</sup>

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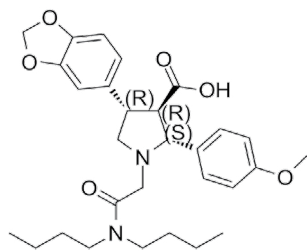


Figure 1: Structure of atrasentan

## MATERIAL AND METHODS

### Chemicals and Standards

Pharma Life Research Lab (Mumbai, India) provided atrasentan and verapamil. Rankem, Biosolve, and SD Fine Chem supplied the ammonium formate, methanol, and formic acid, respectively.

### Instrumentation and Optimized Experimental Conditions

Utilizing a Waters Acquity SDS LC system in conjunction with a Quattro Premier XE mass spectrometer outfitted with electrospray ionization (ESI), the LC-MS analysis was performed. Mass Lynx 4.1 SCN 805 was the software utilized for data collection and processing. The stationary phase utilized is Agilent Zorbax XDB C18, which has the following dimensions:  $2.1 \times 50$  mm and particulates with a size of  $5 \mu\text{m}$ .

The chromatographic separation was achieved by utilizing Shimadzu pumps and an Autosampler for sample injection. The column ( $2.1 \times 50$  mm,  $5 \mu\text{m}$ ) of the Agilent Zorbax XDB C18. To maximise the mobile phase, acetonitrile (0.1% formic acid) and 5 mM ammonium formate were combined 70:30% v/v. The experiment used 0.150 mL/min isocratic elution. The injection volume was  $10 \mu\text{L}$ , and the experiment lasted 3.0 minutes. The column temperature was  $40 \pm 5^\circ\text{C}$ , while the auto-sampler was held at  $5 \pm 3^\circ\text{C}$ .

### Preparation of Atrasentan Standard and Working Solutions

To prepare the stock solution, 10 mg of atrasentan was dissolved in 1% ammonia in acetonitrile. In order to attain a concentration of  $1000 \mu\text{g/mL}$ , the solution was reduced to 10 mL in a volumetric flask utilizing the identical solvent. A constant temperature of 2 to  $8^\circ\text{C}$  was applied to the solution. Utilizing diluents, stock solutions were diluted to acceptable concentrations. When agitated into plasma, these attenuated solutions generated standards for the calibration curve and quality control samples for subsequent use. All dilutions were produced, with the exception of those involving the mobile phase.

### Stock Solution Preparation of Internal Standard

In the mobile phase, a stock solution of internal standard verapamil was dissolved at a concentration of 10 mg. The solution was subsequently prepared by adding 10 mL of liquid to a volumetric flask to achieve the required volume. Attained a concentration of  $1000 \text{ g/mL}$ . The solution was stored between

2 and  $8^\circ\text{C}$  in a refrigerator. The aforementioned stock solution was diluted adequately prior to its utilization in the formulation of efficacious internal standard solutions.

### Plasma Sample Preparation

For the preparation of plasma, human blood samples were extracted and subsequently transferred into polypropylene containers that were supplemented with K2-EDTA. Following 15 minutes of 8500 rpm centrifugation in each tube, the supernatant was withdrawn into an individual tube. Prior to its subsequent utilization, the supernatant was supplemented with 1-mL of acetonitrile and left undisturbed for a duration of 10 minutes to facilitate the precipitation of plasma proteins.

### Spiked Human Plasma Procedure

The technique used to extract IS and atrasentan from human plasma was called liquid-liquid extraction. To complete the process, we filled labeled polypropylene tubes with aliquots of  $100 \mu\text{L}$  of plasma sample and  $20 \mu\text{L}$  of internal standard. The tubes were then momentarily vortexed. The mixture was vortexed once  $20 \mu\text{L}$  of diluent was added. After that, a vortexer was used to thoroughly mix the mixture with  $20 \mu\text{L}$  of a 0.2% formic acid solution. After that, add 5 mL of ammonium acetate to the mixture and use a reciprocating shaker set to 500 rpm to shake it for 30 minutes. The materials were centrifuged for ten minutes at 2000 rpm and  $5^\circ\text{C}$ . After that, 5.0 mL of the organic layer supernatant was carefully transferred to glass test tubes that had already been labeled. After that, the test tube contents were dried out using a turboVap that was set to  $40^\circ\text{C}$ . About  $1000 \mu\text{L}$  of mobile phase, comprising of 70% acetonitrile, 0.1% formic acid, and 30% ammonium formate, was used to reconstitute the samples. The reconstituted sample was then injected into the HPLC system fitted with an MS-MS detection device in an amount of  $10 \mu\text{L}$ .

### Linearity

Linear calibration curves covered atrasentan concentrations between 2 and  $1000 \text{ ng/mL}$ . The correlation was rather strong on average, at 0.999. The peak area of analyte: that of IS was calculated to quantify

### Precision and Accuracy (P and A)

Analyzing six duplicates having atrasentan at four distinct QC levels was done to assess the intra-assay accuracy and precision. The four sets of QC samples from four distinct runs were analyzed to evaluate the inter-assay precision. With the exception of LLQC, which should be between 80 to 120% for accuracy and below 20% of RSD, the findings must meet the following requirements in order to be considered acceptable accuracy between 85 to 115% of real values, and precision around  $\pm 15\%$  of RSD

### Recovery

The quantification of an analyte's recovery in an analytical procedure serves as a metric for evaluating the effectiveness with which it is retrieved throughout the sample processing stage. The calculation of the mean recovery percentage involved comparing the average peak area of six replicated

quality control samples extracted from plasma, at high, middle, and low concentrations, with the average peak area of six replicated quality control samples that were not extracted, also at high, middle, and low concentrations.

**Matrix Effect**

Two sets of extracted blank plasma samples are obtained; each set has six tubes containing plasma from six distinct batches. An equal aqueous concentration of LQC is used to reconstitute one set of tubes, while an equivalent aqueous concentration of HQC is used in the other set. We refer to these samples as post-spiked samples. These samples undergo analysis in conjunction with corresponding aqueous LQC and HQC samples.

**Stability**

Analyte stability under various situations that may arise during sample analysis was assessed by stability tests conducted on both stock solutions and plasma samples. The stability of the stock solution was evaluated by comparing the area response of stability samples, which comprised analytes and an internal standard, with a sample drawn from freshly prepared stock solutions.

**RESULTS AND DISCUSSION**

**Method Development**

To enhance the LC-MS method, a number of mobile phase compositions and characteristics were looked at. An Agilent Zorbax XDB C18 column (2.1 × 50 mm, 5 μm) was utilized to effectively separate atrasentan, ensuring acceptable peak symmetry. Acetonitrile, 0.1% formic acid, and 5 mM ammonium formate were combined in a 70:30% v/v ratio to create the mobile phase. Using a flow rate of 0.150 mL/min and an injection volume of 10 μL, the separation was carried out in positive ion mode (API 4000). The analysis took four minutes to finish in total. The detection procedure is executed utilising positive ion mode electrospray ionization (ESI) mass spectrometry under atmospheric pressure. In Figures 2 and 3, atrasentan, the precursor to product ion transitions used for quantification were m/z 511.623 to 354.15 and for the internal standard, verapamil, m/z 455.22 to 165.02. The retention times for atrasentan and verapamil were found to be 1.68 and 0.96 minutes, respectively. Figures 4 to 6 depict a standard chromatogram showcasing blank plasma, atrasentan, and verapamil (Internal standard).

*Selectivity*

There was no observed response in the blank samples, suggesting that there was no interference from plasma at room temperature. This observation is consistent with what was seen in the chromatograms for atrasentan.

*Carryover*

When atrasentan was injected after HQC, the response in the blank chromatogram was completely absent. Because the analyte peak response was less than 20% of the lower limit of quantification (LLOQ), the suggested method does not have a carryover issue.

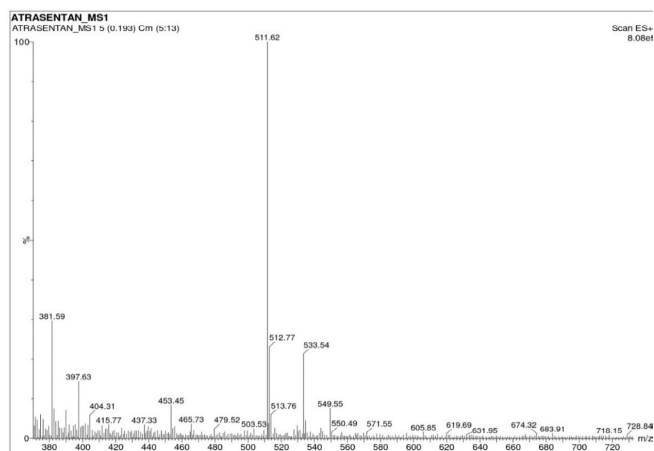


Figure 2: Mass fragmentation pattern of atrasentan

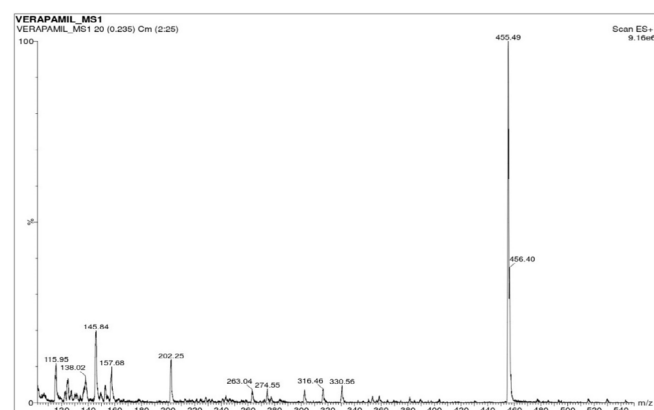


Figure 3: Mass fragmentation pattern of verapamil

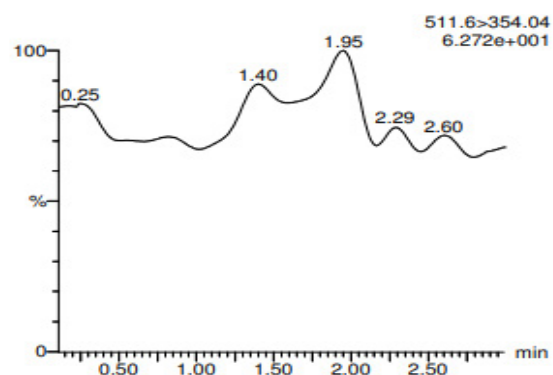


Figure 4: Blank plasma chromatogram

*Sensitivity*

The peak area of the blanks at the LLOQ was greater than five times that of the analytes. For atrasentan, the mean accuracy across the six injections performed at the LLOQ was 102.62%. For atrasentan, the precision (%CV) values were 0.48%. The developed method demonstrated sensitivity even at a low concentration of 1.100 ng/mL (LLOQ). Figure 7 displays the chromatographic representation of the lower limit of quantification (LLOQ) when an internal standard (IS) is used.

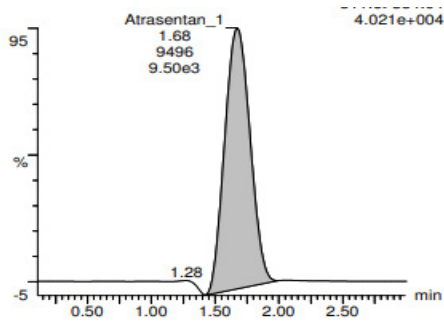


Figure 5: Standard chromatogram of atrasentan

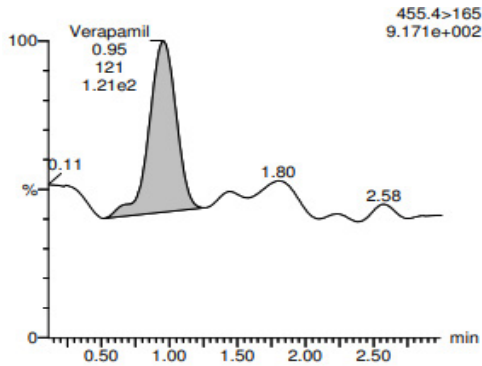


Figure 6: Internal Standard chromatogram of verapamil

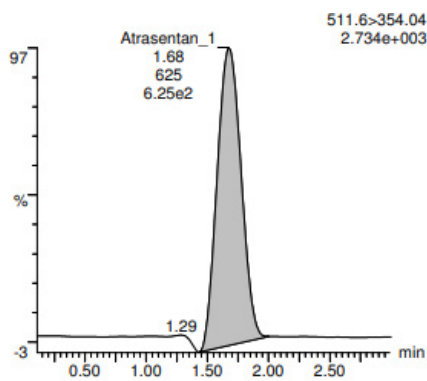
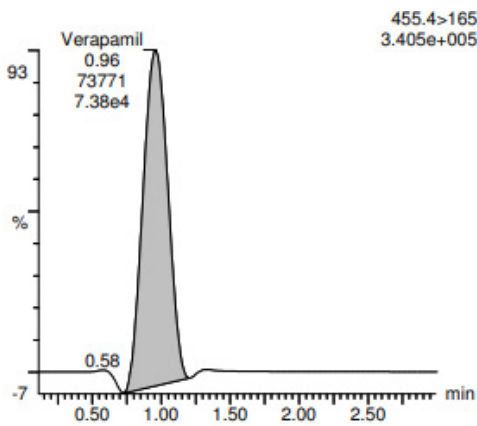


Figure 7: LLoQC chromatogram of atrasentan

Table 1: Linearity results of atrasentan

Final conc. in ng/mL	Atrasentan peak area
0	0
2	53
12	218
50	1057
200	4270
500	9988
800	15638
1000	19175
Slope	19.296
Intercept	115.38
R <sup>2</sup>	0.9994

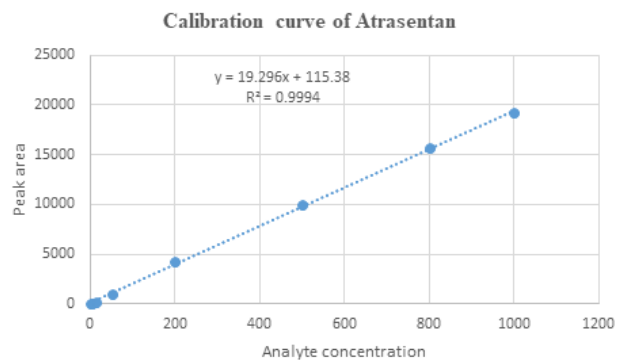


Figure 8: Calibration curve of atrasentan

*Linearity*

The calibration curve associated with atrasentan demonstrated linearity across the concentration range of 2 to 1000 ng/mL, as depicted in Figure 8. The results of the least square regression analysis indicated that a straight line could effectively represent the data points, suggesting a constant proportionality and minimal data dispersal. Table 1 shows that the correlation coefficient (r) for atrasentan was 0.9994.

*Precision and accuracy*

The purpose of this study was to evaluate the accuracy and precision of the intra-assay by analyzing six duplicates of atrasentan at four unique quality control (QC) levels. The inter-assay precision was assessed by analyzing the four sets of quality control samples obtained from four separate runs. Except for LLQC, which should have an accuracy range of 80 to 120% and an RSD below 20%, the results must satisfy the following criteria to be deemed acceptable: accuracy within the range of 85 to 115% of the true values and precision within  $\pm 15\%$  of the RSD. The findings are tabulated in Table 2.

*Extraction recovery*

The QC levels for atrasentan indicate that the extraction recovery was between 97.44 and 98.25%. This illustrates that the sample preparation approach is extremely successful for drug analysis since it exhibits a high analyte extraction efficiency.

**Table 2:** Precision and accuracy findings of atrasentan

Acquisition	HQC	MQC	LQC	LLQC
	Nominal conc. (ng/mL)			
	780.00	480.0	3.00	1.100
	Analyte peak area			
	14684	9589	67	28
	14689	9585	59	26
	14672	9583	68	28
	14669	9578	69	29
	14678	9582	70	25
	14675	9577	64	29
n	6	6	6	6
Mean	14677	9582	66.16	27.5
SD	7.52	4.45	4.07	1.64
%CV	0.05	0.04	6.15	5.9
%Mean Accuracy	93.85	96.44	111.66	96.39

**Table 3:** Stability summary of atrasentan

Stability	QC	Mean ± SD	%Stability	%CV
Bench top stability (24 hours)	LQC	64 ± 4.38	108	6.8
	HQC	14686 ± 3.544	100.01	0.02
Freeze and thaw stability	LQC	61.5 ± 3.33	102.81	5.41
	HQC	14685 ± 2.250	100.00	0.015
Autosampler stability (2–8°C (64 hours)	LQC	61.5 ± 3.33	102.81	5.41
	HQC	14685 ± 2.250	100.00	0.015
Short-term stability (9.5 hours)	LQC	68.12 ± 3.120	100.12	2.25
	HQC	14645 ± 2.014	101.21	0.147
Long term stability at -30°C (64 days)	LQC	59.6 ± 4.08	99.28	2.156
	HQC	14688 ± 6.84	101.25	0.127

**Matrix effect**

Through comparison of the responses of extracted MQC samples (n = 6) with those of analyte from fresh samples at the same dosage, the matrix of plasma components over the ionization of the analyte was found. The proposed technique of matrix impact was evaluated using chromatographically processed plasma samples. Atrasentan has a precision (%CV) of 0.04 at HQC and 2.8 at LQC.

**Stability**

The mean of six replicates (n = 6) was used to assess the stability of the long-term stability and short-term stability. Both the drug and the internal standard (ISTD) exhibited mean stability values that fell within the permissible range of ± 10%. We conducted bench-top stability testing using six replicates (n = 6) and calculated the mean. The coefficient of variation (%CV) and mean stability were found to be within the acceptance criteria, as shown in Table 3.

**CONCLUSION**

The goal of this research is to create a bioanalytical technique for atrasentan. The technique utilized to identify and quantify atrasentan in human plasma is extremely specific, sensitive,

and straightforward. A simple liquid-liquid Extraction method is used in the sample preparation procedure. Because the approach is extremely repeatable, it provides a low-cost option. The method that was developed was determined to be acceptable based on various parameters. The analysis as a whole demonstrates promising prospects for the future. This study is expected to make a substantial contribution to the field of bioanalytical research. Pharmaceutical pharmacokinetics, bioequivalence, and quality control laboratories are a few of the applications where the bioanalytical method that was devised in this study holds promise for future success.

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