

## Application Note

# Validation of a Bioanalytical Method for Measurement of Alprazolam in Human Plasma

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## Abstract

Using the excellent reproducibility of the Waters Alliance HT HPLC System coupled to the highly sensitive and selective Micromass Quattro micro we were able to develop and validate a method for the bioanalytic study of alprazolam which met FDA guidelines for bioanalytical method validation. All data were acquired using a secure installation of MassLynx v4.0 Software with the QuanLynx Application Manager which are designed to work in a 21 CFR 11 compliant environment.

## Benefits

The method shows acceptable precision and accuracy throughout the calibration range which resulted in good linearity

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## Introduction

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The requirements of the regulatory bodies, such as the FDA,<sup>1</sup> are very stringent for the bioanalytical laboratory. This makes the need for highly selective and sensitive analytical methodologies for the quantitative evaluation of drugs, very important in drug discovery development.

Obtaining low levels of detection with very selective and sensitive methodologies is the key for monitoring the therapeutic dose level in order to ascertain the efficacy of the drug. This report describes the validation of a high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) procedure for the measurement of alprazolam in human plasma.

Tandem MS with liquid chromatography offers an integrated solution for bioanalytical studies as it generates accurate and unambiguous data. Using MassLynx v4.0 Software, the data can be obtained in a 21 CFR part 11 compliant environment. The software maintains the integrity of the data by controlling the whole process from generation of tuning parameters (initial stages of method development) to the storage and archiving of the final validation report.

Bioanalytical method validation has a variety of important parameters, which are used for the quantitative measurements of analytes in a given biological matrix, such as plasma. These parameters are crucial for regulatory compliance on the validation of bioanalytical methods. These parameters include:

Accuracy – the closeness of the mean test results obtained by the method to the true concentration of the analyte.

Precision – the closeness of individual measures of an analyte, after multiple analysis.

Selectivity – the ability of the method to differentiate and quantify the analyte in the biological matrix, from any endogenous material.

Sensitivity – to routinely give easily determinable values at the lowest end of a calibration series.

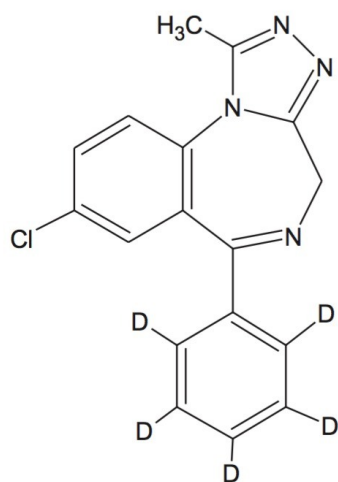
Reproducibility – the precision of a method under same conditions over a period of time.

Stability – this is a function of storage conditions, chemical properties, the matrix and container system.

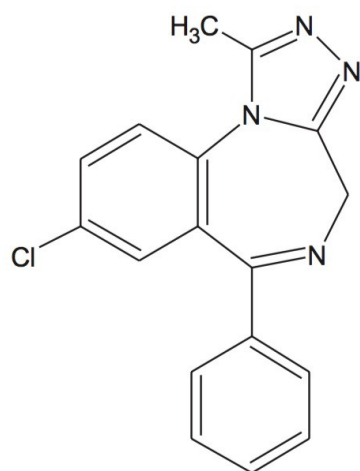


*The Waters Alliance LC-MS/MS System*

For validation purposes the accuracy and precision are determined with mean values within  $\pm 15\%$  of theoretical values, except at the lower limit of quantification (LLOQ) where it is  $\pm 20\%$ .



*Figure 1. Alprazolam.*



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Figure 2. Alprazolam-D5.

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## Experimental

### Analytical Equipment and Instrumentation

Waters Micromass Quattro micro Tandem Mass Spectrometer

Waters 2795 Separations Module

MassLynx Data Acquisition System

QuanLynx Application Manager

### Materials

Alprazolam, alprazolam-D5 (internal Standard), acetonitrile, ammonium acetate, water, blank human plasma from Sigma-Aldrich Chemical Co. Poole, UK.

### Analysis Conditions

Mobile Phase

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Mobile phase A – (95% 5mM ammonium acetate, pH, 5% acetonitrile) 0.04% acetic acid

Mobile phase B – (5% 5mM ammonium acetate, pH, 95% acetonitrile) 0.04% acetic acid

Flow – 400  $\mu$ L/min

Gradient Timetable -	Time	B%
	0.00	28.0
	0.50	28.0
	5.00	98.0
	5.50	98.0
	5.60	28.0
	10.0	28.0

## LC Columns and Conditions

Column = YMC-Pack ODS-AQ (50 x 2.1 mm id)

## Preparation of Standard Solutions

Alprazolam primary stock solution (1 mg/mL) and working standard solutions A primary stock solution of alprazolam was prepared at a concentration of 1 mg/mL by accurately weighing out 5 mg of alprazolam into a flask and dissolving in 5 mL of mobile phase.

Internal standard primary stock solution (100 ng/ $\mu$ L) Internal standard solution was prepared by diluting the stock solution 1 in 10 with acetonitrile/water, 50/50, (v/v).

Alprazolam secondary stock solutions (100 ng/ $\mu$ L, 1 ng/ $\mu$ L and 100 pg/ $\mu$ L) The 1 mg/mL solution was diluted 1 in 10 with 50/50 (v/v), acetonitrile/water to produce a working solution. Further dilutions were made to produce the 1 ng/ $\mu$ L and 100 pg/ $\mu$ L solutions.

## Preparation of Human Plasma Calibration Standards

Aliquots of alprazolam working standard solutions were added to blank human plasma on the day of the analysis to produce a calibration line at the concentrations stipulated below.

Concentration of alprazolam standard solution (ng/ $\mu$ L)	Volume of standard solution ( $\mu$ L)	Volume of internal standard ( $\mu$ L)	Volume of plasma ( $\mu$ L) + 500 $\mu$ L of acetonitrile	Final concentration (pg/ $\mu$ L)
100	50	10	440	5000
100	10	10	480	1000
100	5	10	485	500
1	100	10	390	100
1	50	10	440	50
1	10	10	480	10
0.1	50	10	440	5

### Preparation of Quality Control Samples

Quality control (QC) samples were prepared to assess the precision of the analytical method, the stability of the alprazolam in plasma and the recovery. The QC samples were prepared independently using stock solutions from the working standards. The samples were prepared according to the table below.

The QC samples were divided into aliquots of 25  $\mu$ L and placed into sample vials. The initial prepared samples were analysed immediately and the remaining samples were stored at -22 °C prior to analysis.

Standard concentration used (ng/ $\mu$ L)	Volume of standard solution ( $\mu$ L)	Volume of internal standard ( $\mu$ L)	Volume of plasma ( $\mu$ L) + 500 $\mu$ L of acetonitrile	Final concentration (pg/ $\mu$ L)
0.1	10	10	480	1
1	30	10	460	30
10	30	10	460	300
100	30	10	460	3000

### Preparation of Standard Mixture Solution (System Suitability)

A standard mixture solution was prepared containing 100 pg/ $\mu$ L of both alprazolam and alprazolam-D5 in acetonitrile/water (50/50, v/v).

## Sample Extraction Procedure

1. The method consisted of mixing human plasma, varying amounts depending on the standard used (see table) with internal standard (alprazolam-D5 at 100 pg/ $\mu$ L, 10  $\mu$ L).
2. The resulting mixture was vortex mixed (ca 10 seconds) and centrifuged (ca 3000 rpm, ca 3 minutes).
3. The supernatant was placed into a glass vial and injected onto a YMC-Pack ODS-AQ (50 x 21 mm id) HPLC Column.
4. A 10  $\mu$ L injection of the extract was run by gradient reversed-phase chromatography with a mobile phase comprising of 5 mM ammonium acetate (5%):95 acetonitrile at a flow rate of 0.4 mL/minute.

## Calculations

Calibration lines were prepared on the basis of peak area ratios versus concentration of alprazolam in matrix. A  $1/x^2$  weighted least squares regression analysis of the data was used to calculate the slope, y-intercept and correlation coefficient.

## Validation Parameters

### Linearity of the matrix standard curve

Standard curves were constructed from replicate analyses of eight concentrations over the range 1 to 5000 pg/ $\mu$ L.

The linearity of the matrix standards was determined by plotting peak area ratios against concentration. A weighting of  $1/x^2$  was applied in order to improve the accuracy of measurements at low levels. The information obtained included the gradient (slope), y-intercept, correlation coefficient (r), and the back-calculated calibration standard concentrations.

### Precision

The precision of the assay was evaluated by the coefficient of variation (CV) within and overall from the batch analyses. A total of four different QC samples were prepared at the LOQ, low, medium and high concentrations over the calibration range. A total of eight QC samples were analyzed (two at each concentration) on a daily basis.

### Accuracy

The accuracy was determined by measuring the QC samples and comparing them with the theoretical concentrations. The following formula was used for the calculation of the relative error (RE):

$$\text{RE (\%)} = (\text{Mean calculated conc.} - \text{theoretical conc.}) \times 100 (\%)$$

### Sensitivity (limit of quantification)

The LOQ of the assay was the lowest concentration of the calibration series with a precision of  $\pm 20\%$ .

### Specificity

This was ascertained by comparing the blank human plasma with the spiked plasma in order to see any interfering peaks at the same retention times and mass transition.

### Stability of test substance

This was monitored by the freeze thaw cycles. As the first QC samples were prepared they were analyzed immediately. Then the remaining samples were divide up into QC samples and freeze thaw QC samples. The freeze thaw QC samples were taken out of a freezer (at  $-22\text{ }^{\circ}\text{C}$ ) and left on the bench for 2 hours (sufficient time to defrost). They were then analyzed and returned to the freezer. This was done on day one, two and three of the validation procedure.

### System suitability

The standard mixture was injected prior to the calibration series and the peak areas, ratios and shape were monitored. Action such as changing mobile phase or column was taken if any significant change was observed.

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## Results and Discussion

### Linearity of the matrix standard curves

The relationship of the peak area ratio of alprazolam and the alprazolam-D5 internal standard with concentration was linear (mean  $r=0.9968$ ,  $n=8$ ) over the calibration range 1 to 5000 pg/ $\mu\text{L}$ . The back-calculated concentrations for each calibration point expressed as relative error were between  $-9.0\%$  and  $2.7\%$  over the calibration range. Examples of the calibration line are shown in Figure 3 and typical chromatograms for each calibration point are shown in Figures 4A-4D.



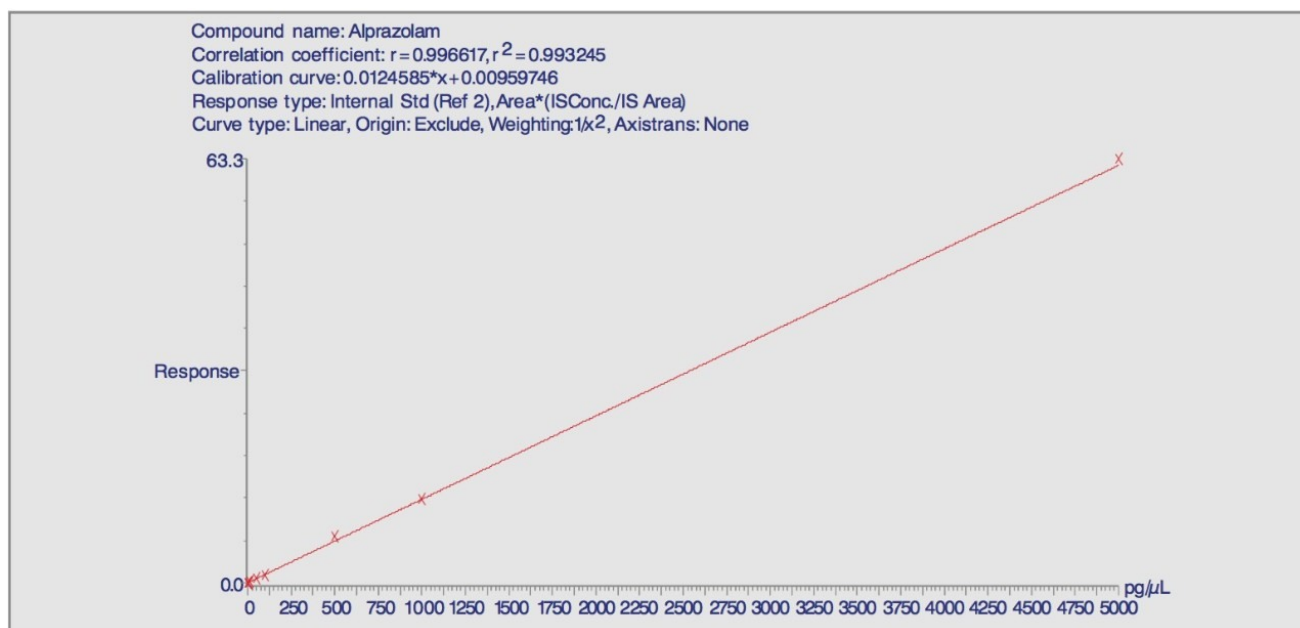


Figure 3. A typical representative calibration line over the linear range 1 to 5000 pg/μL.

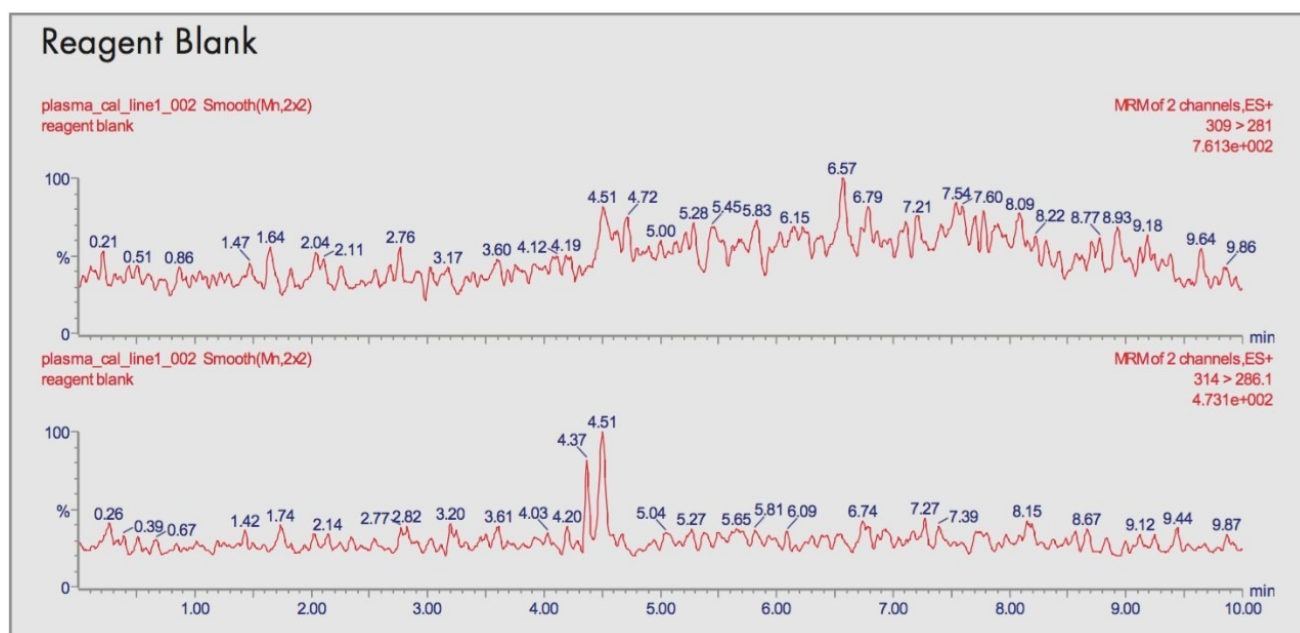


Figure 4A. Representative chromatograms of alprazolam standard extracts from human plasma.

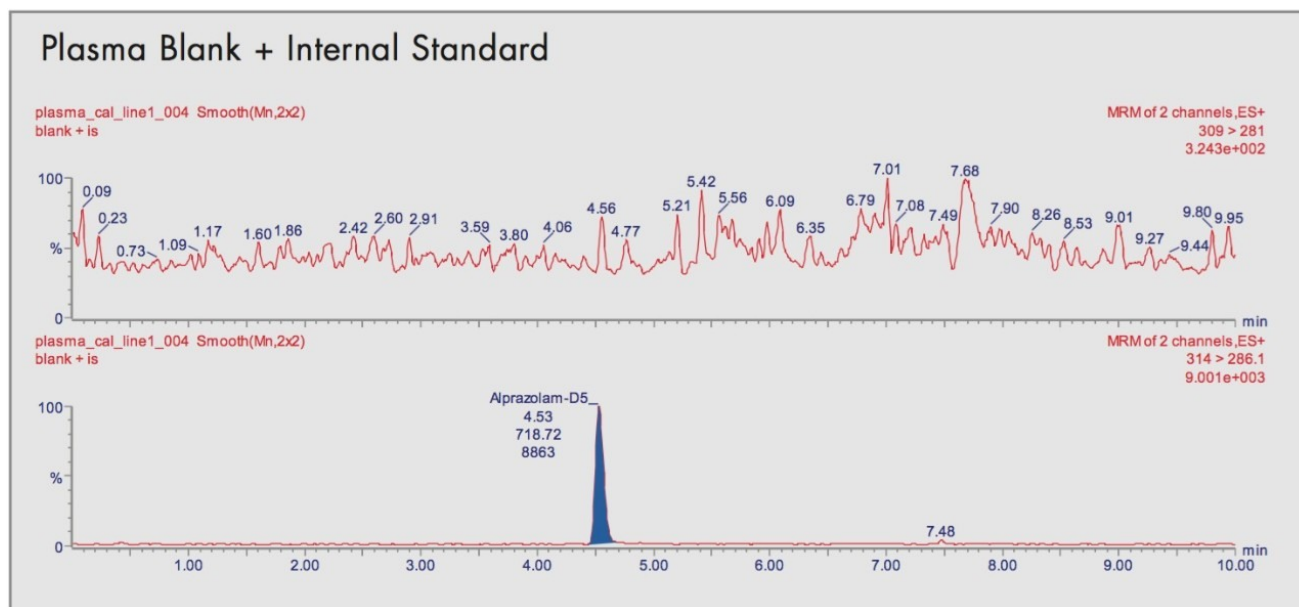


Figure 4B. Representative chromatograms of alprazolam standard extracts from human plasma.

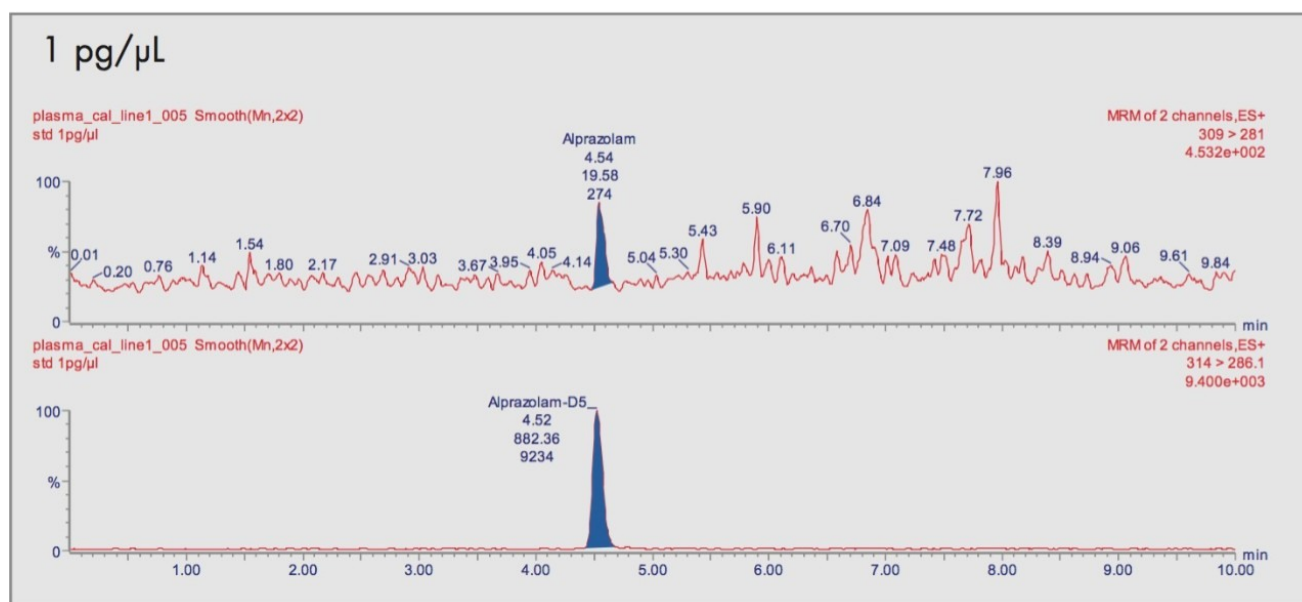


Figure 4C. Representative chromatograms of alprazolam standard extracts from human plasma.

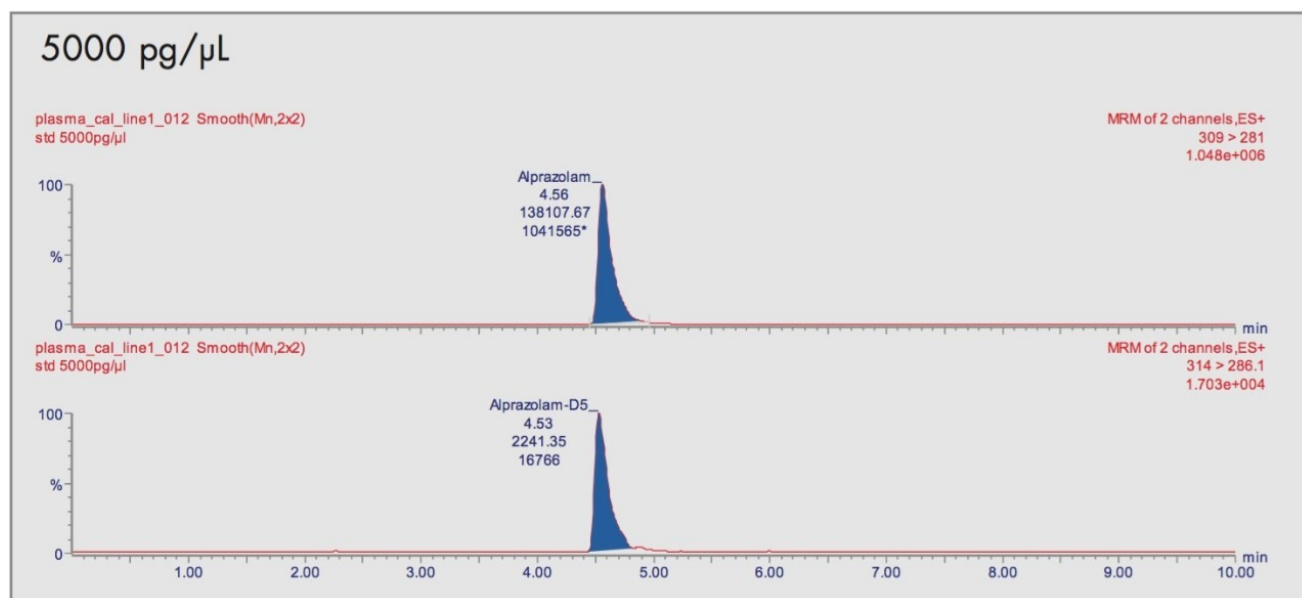


Figure 4D. Representative chromatograms of alprazolam standard extracts from human plasma.

The daily calibration measurements are summarized in Table 1.

	Back-calculated values of concentration							
			Concentration (pg/ $\mu$ L)					
Calibration Number	1	5	10	50	100	500	1000	5000
1	1.01	4.61	10.65	48.58	88.33	566.64	998.66	5078.62
2	1.11	4.50	8.90	49.00	100.47	553.65	1062.05	5226.43
3	0.94	4.56	9.98	51.80	95.81	520.97	985.06	5155.57
4	n/a	4.47	9.96	47.35	103.47	544.34	1030.02	5478.04
5	1.03	3.80	9.91	43.87	93.11	535.57	1075.94	4704.88
6	1.04	4.38	9.09	48.92	109.20	464.33	1105.13	n/a
7	n/a	3.78	8.86	49.01	107.90	495.52	1060.21	5345.89
mean	1.03	4.30	9.62	48.36	99.76	525.86	1045.30	5164.91
sd	0.06	0.36	0.68	2.39	7.75	35.59	42.93	265.99
CV(%)	<b>5.95</b>	<b>8.27</b>	<b>7.06</b>	<b>4.94</b>	<b>7.77</b>	<b>6.77</b>	<b>4.11</b>	<b>5.15</b>

Calibration Number	Slope	Intercept	r	r <sup>2</sup>
1	0.0125	0.0096	0.9966	0.9932
2	0.0116	0.0041	0.9972	0.9944
3	0.0127	0.0064	0.9972	0.9944
4	0.0108	0.0058	0.9964	0.9929
mean	0.0119	0.0065	0.9969	0.9937
sd	0.0009			
CV(%)	7.3050			

Table 1.

Overall batch precision measurements were 7.5% at 1 pg/μL, 6.1% at 30 pg/μL, 2.6% at 300 pg/μL and 8.2% at

3000 pg/μL (Table 2).

## Overall batch precision and accuracy measurements of alprazolam in human plasma

Overall-Batch precision and accuracy of Alprazolam in Human Plasma									
Concentration spiked (pg/μL)	Concentration Found(pg/μL)		Accuracy (%)		mean	sd	CV(%)	n	RE(%)
	n/a	n/a	n/a	n/a					
1	1.07	1.12	107	112					
	1.01	0.91	101	91					
	0.98	0.96	98	96					
					1.01	0.08	7.56	6.00	0.83
30	25.51	24.85	85.03	82.83					
	28.12	27.22	93.73	90.73					
	26.34	27.90	87.80	93.00					
	30.13	27.29	100.43	90.97	27.17	1.65	6.07	8.00	-9.43
300	260.26	261.41	86.75	87.14					
	271.99	271.36	90.66	90.45					
	270.77	277.45	90.26	92.48					
	274.94	280.23	91.65	93.41	271.05	7.08	2.61	8.00	-9.65
3000	2988.76	2907.15	99.63	96.91					
	3124.86	3122.90	104.16	104.10					
	2983.14	2869.02	99.44	95.63					
	3001.50	3664.06	100.05	122.14	3082.67	251.62	8.16	8.00	2.76

sd  
CV

standard deviation  
Coefficient of variation

RE  
n/a

Mean relative error  
excluded from linear regression and  
calculation of mean due to injection  
error

Table 2.

## Sensitivity (Limit of Quantification)

The already defined limit of quantification (LOQ), was 1 pg/μL in plasma using the lowest calibration standard. At this level the back-calculated concentrations gave a mean value of 1.03 pg/μL with a CV of 5.9%.

## Specificity

In extracts of blank human plasma there were no interfering peaks at the same retention times as the alprazolam



and the internal standard. Typical chromatograms are shown in Figure 5 for the blanks and comparisons with the calibrants show no carry-over between injections.

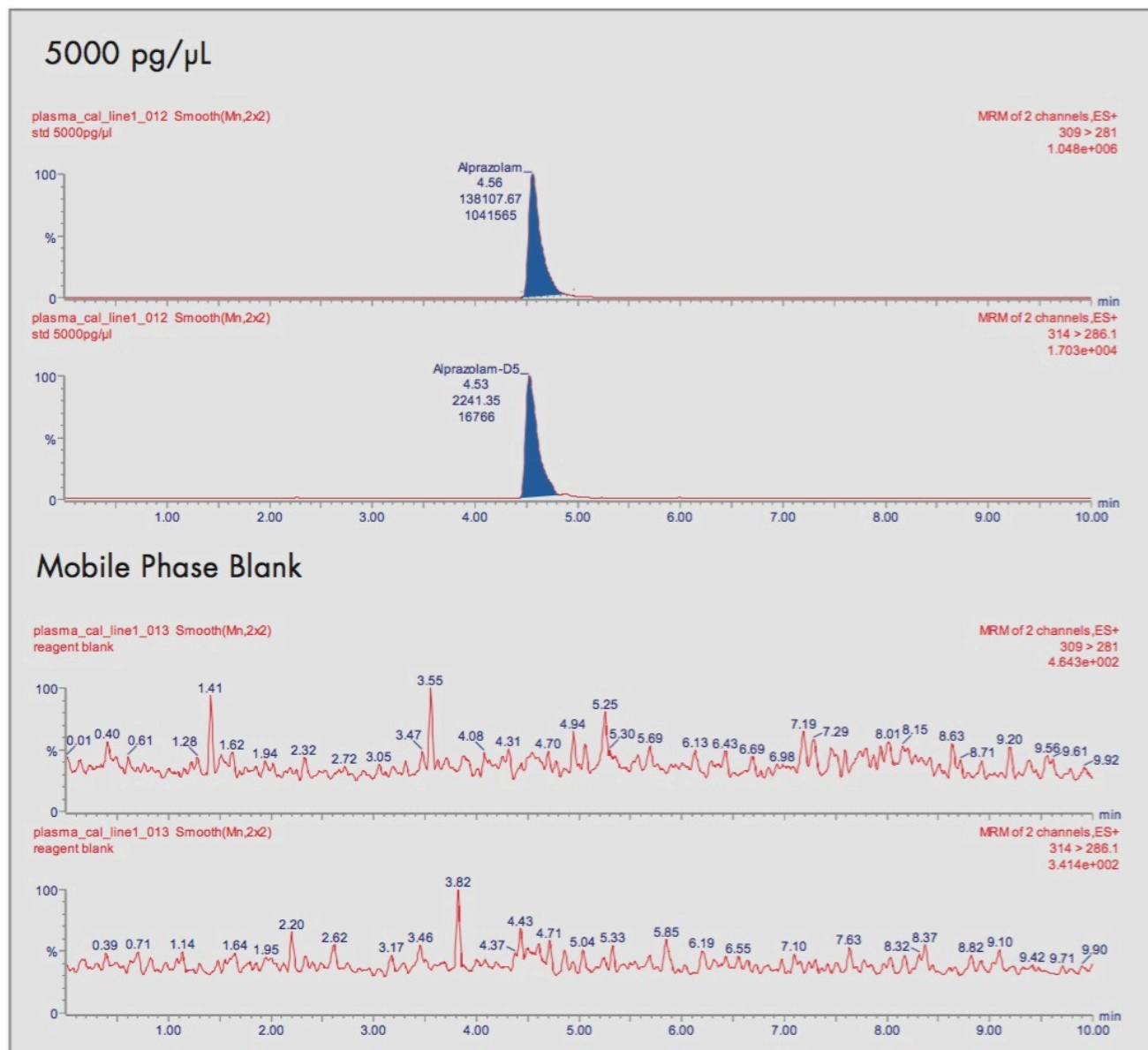


Figure 5. Representative chromatograms demonstrating the absence of carry-over.

## Stability of the test substance

Alprazolam was shown to be stable in protein-precipitated human plasma at ambient room temperature

(nominally +22 °C) for up to 2 hours, stored frozen at nominally -22 °C and following 3 freeze/thaw cycles (Table 3).

Stability measurements of alprazolam in human plasma							
		Freeze Thaw Cycles					
Concentration (pg/μl)		Day 1	Day 2	Day 3	mean	sd	cv
30		27.22	27.90	27.29	27.47	0.37	1.36
300		271.36	277.45	280.23	276.35	4.54	1.64
3000		3122.90	2869.02	3664.06	3218.66	406.08	12.62
sd CV	standard deviation Coefficient of variation	RE n/a		Mean relative error excluded from linear regression and calculation of mean due to injection error			

Table 3.

The results showed that over a calibration range of 1 to 5000 pg//L the linearity was in excess of 0.99, (Figure 3) with all standards within the range of -9 to 2.7% relative error. This is well within the accepted criteria of  $\pm 15\%$  (20% at the lower limit of quantitation) as defined by the FDA.

The overall batch precision and accuracy for the QC's at 1, 30, 300 and 3000 pg/μL were in the range of 6.1 to 8.2%, again well within the  $\pm 15\%$  defined. The defined limit of quantification was maintained at 1 pg/μL with a mean back-calculated concentration of 5.9%. This highlights a very precise and accurate developed method that maintained the integrity of the standards throughout the validation.

The freeze thaw cycles for the QC's at 30,300 and 3000 pg/μL were within the range 1.36 to 12.62% (coefficient of variation).

Overall the data showed excellent results from the initial method development to the final validated data. All the extracted standards and QC's (and freeze thaw QC's) gave excellent individual results well within the strict criteria

set by the FDA of  $\pm 15\%$ . The overall batch precision and accuracy also fit well within the regulatory guidelines.

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## Conclusion

It is concluded that the bioanalytical method for the measurement of alprazolam in protein-precipitated human plasma has been successfully validated over the calibration range 1 to 5000 pg/ $\mu$ L. The method shows acceptable precision and accuracy throughout the calibration range which resulted in good linearity. The limit of quantification was 1 pg/ $\mu$ L with an injection volume of 10  $\mu$ L.

Alprazolam was stable in protein precipitated human plasma at room temperature for up to 2 hours, stored frozen at nominally -22 °C and following 3 freeze/thaw cycles.

Using the excellent reproducibility of the Waters Alliance HT HPLC System coupled to the highly sensitive and selective Micromass Quattro micro we were able to develop and validate a method for the bioanalytic study of alprazolam which met FDA guidelines for bioanalytical method validation. All data were acquired using a secure installation of MassLynx v4.0 Software with the QuanLynx Application Manager which are designed to work in a 21 CFR 11 compliant environment.

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## References

1. Guidance for Industry - Bioanalytical Method Validation, US Department of Health and Human Services, FDA, May 2001.

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## Featured Products

Alliance HPLC <<https://www.waters.com/514248>>

MassLynx MS Software <<https://www.waters.com/513662>>

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720000521, September 2003

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