A High Sensitivity Method for the Quantification of Fluvastatin in Plasma Using an Agilent 1290 Infinity Binary LC and an Agilent 6490 Triple Quadrupole Mass Spectrometer

Application Note

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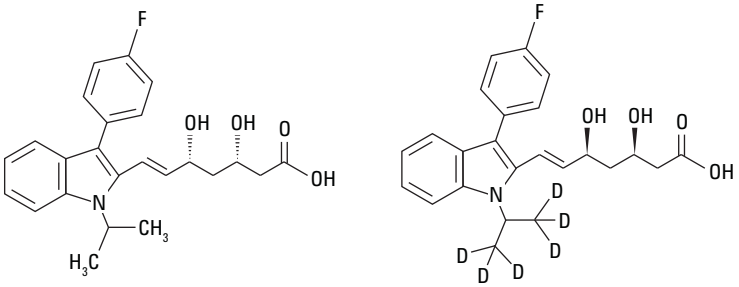
Abstract

This Application Note describes the development of an LC/MS bioanalytical method for the quantification of fluvastatin in human plasma using an Agilent 1290 Infinity Binary LC and an Agilent 6490 Triple Quadrupole Mass Spectrometer. A deuterated analog of fluvastatin was used as the internal standard. The calibration curve was developed in the range of 0.2 to 50 ng/mL, and the limit of detection was 0.1 ng/mL. The calibration standards and three quality control samples showed excellent recoveries and precision. The method exhibited excellent intra- and interday reproducibility and negligible carryover. The instrumentation and the developed method are suitable for routine bioanalytical measurements of fluvastatin in human plasma.



Fluvastatin Fluvastatin-d6

Figure 1. Compounds used in this study.

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Introduction

Highly sensitive analytical techniques are required for the quantification of pharmaceuticals in small volumes of complex matrixes such as blood plasma. This becomes even more important when developing bioanalytical methods for compounds that are rapidly metabolized or eliminated from the body resulting in very low levels in the study samples. An Agilent 1290 Infinity Binary LC and an Agilent 6490 Triple Quadrupole Mass Spectrometer was used to develop an accurate and precise bioanalytical method for the quantification of fluvastatin in a sample volume of 200 µL, using 20 ng/mL of Fluvastatin-d6 as the internal standard.

Experimental

Chemicals

Fluvastatin, human plasma (lyophilized), and ammonium acetate were from Sigma-Aldrich, fluvastatin-d6 was from Clearsynth, and MS grade acetonitrile and methanol were from Fluka. Double distilled water was obtained from a Millipore Milli-Q water purification system.

- Agilent 1290 Infinity Binary LC System comprising:
    - Flexible cube (G4227A)
    - Binary pump (G4220A)
    - Autosampler (G4226A)
    - ALS Thermostat (G1330B)
    - TCC (G1316A)
- Agilent 6490 Triple Quadrupole LC/MS (G6490A)

Sample preparation

Plasma calibration standards

A 160 µL amount of human plasma was spiked with 20 µL of each aqueous fluvastatin standard solution and 20 µL of fluvastatin-d6 solution. The concentration of fluvastatin in the plasma samples ranged from 0.2 ng/mL to 50 ng/mL. These included three QC samples, each with concentrations of 0.75, 5, and 40 ng/mL of fluvastatin.

Plasma blank

A 160 µL amount of plasma was spiked with 40 µL of water.

Plasma blank with internal standard

A 160 µL amount of plasma was spiked with 20 µL of water and 20 µL of fluvastatin-d6 solution. The concentration of fluvastatin-d6 (internal standard) in all plasma samples was 20 ng/mL.

Instrument conditions

LC conditions		
Column	Agilent Eclipse Plus Phenyl Hexyl (RRHD) 2.1 × 50 mm, 1.8 µm (p/n 959757-912)	
Column temperature	40 °C	
Mobile phase A	Ammonium acetate (10 mM)	
Mobile phase B	Acetonitrile	
Flow rate	0.5 mL/min	
Gradient	Time (min)	% ACN
	0.00	30
	0.25	30
	0.90	90
	1.40	90
	1.50	30
	Stop time 2.00	30
	Post time 1.00	
Injection volume	5 µL	
Needle wash	Water for 10 seconds at wash port	
Needle seat backflush using Flexible Cube	3 mL/min methanol for 15 seconds 3 mL/min (80 % ammonium acetate + 20 % ACN) for 15 seconds	
MS conditions		
Gas temperature	200 °C	
Gas flow	12 L/min	
Nebulizer	50 psi	
Sheath gas heater	400 °C	
Sheath gas flow	11 L/min	
Capillary voltage	4,000 V	
Nozzle voltage	300 V	
Delta EMV	200 V	
Fragmentor voltage	380 V	
Pos high pressure RF	150 V	
Neg high pressure RF	90 V	
Pos low pressure RF	60 V	
Neg low pressure RF	60 V	

A simple protein precipitation method was used for sample preparation. The spiked samples were then treated with 600 µL of ice cold acetonitrile to precipitate the proteins, vortexed for 30 seconds, and finally centrifuged at 13,500 rpm for 15 minutes at 6 °C. The supernatant solutions were transferred to new microcentrifuge tubes prior to vacuum concentration. The residues were reconstituted in 200 µL of an 8:2 mixture of mobile phases A and B. To remove any suspended particulates, the solutions were centrifuged once more at 13,500 rpm for 10 minutes at 6 °C. The supernatant solutions were carefully transferred to HPLC vials fitted with vial inserts (made of deactivated glass, p/n 5181-8872) for analysis.

Results and Discussion

Table 1 shows the MRM transitions and MS parameters for fluvastatin and its deuterated analog used as the internal standard in the study.

After optimizing the mass spectrometer parameters and chromatographic separation (Figure 2), the linearity of the method in plasma was evaluated. Triplicate injections of each calibration standard and QC sample were made. The relative response ratios of fluvastatin to fluvastatin-d6 were plotted against fluvastatin concentrations to obtain the calibration curves. Linear curve fitting and (1/x) weighting were used. Correlation coefficient values >0.995 were obtained during three independent evaluations. A representative calibration curve for fluvastatin in plasma using fluvastatin-d6 as internal standard is shown in Figure 3.

Table 1. MRM transitions and MS parameters.

Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)	Dwell time (ms)	Collision cell acceleration voltage (V)
Fluvastatin 412.1	266.2 (Quantifier)	20	50	2
	224 (Qualifier)	22	50	2
Fluvastatin-d6 (ISTD) 418.1	272.1 (Quantifier)	18	50	2

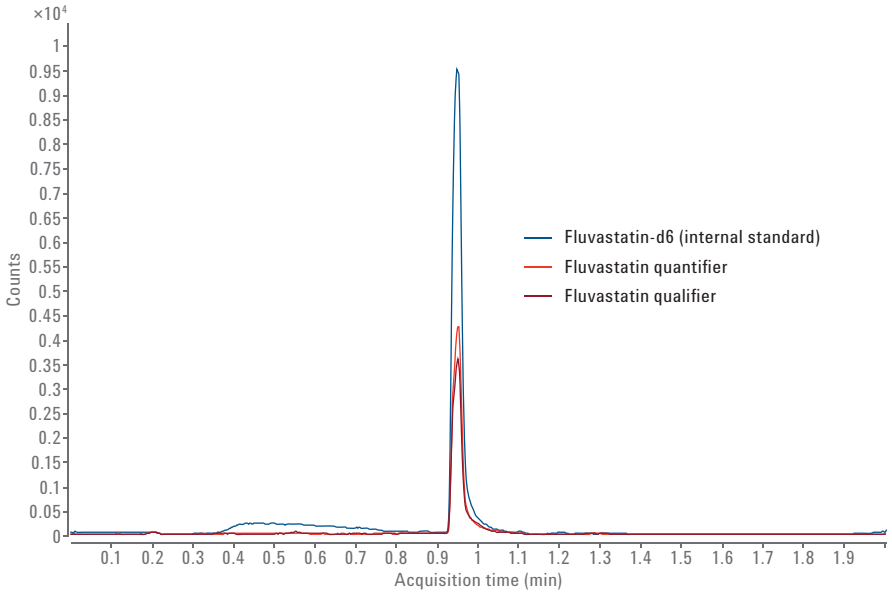

Figure 2. Extracted MRM chromatograms of the fluvastatin quantifier and qualifer transitions as well as of the fluvastatin-d6 MRM transition. Sample contains 5 ppb fluvastatin and 20 ppb fluvastatin-d6.

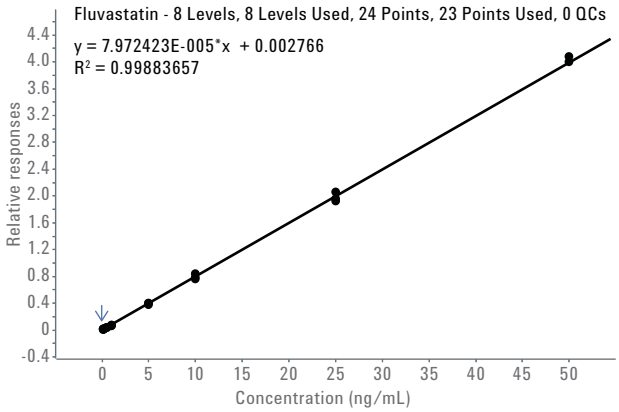

Figure 3. Representative standard curve in plasma. Three replicated injections at each calibration level.

Fluvastatin response linearity in plasma was evaluated on three successive days. Tables 2 and 3 show the back-calculated concentrations, accuracy, and precision values for all calibration and QC standards. In the range of 0.2 to 50 ng/mL, accuracy values are within ± 15 % as recommended by US FDA for bioanalytical methods. Precision values at all levels were also within ± 15 %. Although the relative response for the 0.1 ng/mL level falls on the calibration curve, it was considered the limit of detection (LOD) of the method as its accuracy is outside the tolerance range of 80 to 120 %, recommended by US FDA. The lower limit of quantitation (LLOQ) of the method was 0.2 ng/mL level.

Table 2. Average calculated concentrations (*n = 3) and average accuracy values for each calibration and QC sample. The linearity of fluvastatin relative response in human plasma was evaluated on three successive days.

Experimental concentration (ng/mL)	Calculated concentrations* (ng/mL)					
	Day 1	Accuracy	Day 2	Accuracy	Day 3	Accuracy
0.1	0.15	147.91	0.13	128.59	0.14	139.43
0.2	0.18	87.60	0.19	96.02	0.21	107.04
0.5	0.47	93.53	0.46	91.65	0.46	92.38
1.0	0.87	86.61	0.85	85.44	0.85	84.90
5.0	4.48	89.68	4.91	98.23	4.50	89.94
10.0	8.93	89.30	9.98	99.84	8.43	84.26
25.0	25.96	103.82	24.84	99.34	23.81	95.25
50.0	50.77	101.55	51.93	103.86	53.40	106.80
0.75 (LQC)	0.68	90.99	0.66	87.62	0.63	83.92
5.0 (MQC)	4.66	93.27	4.85	96.91	4.81	96.01
40 (HQC)	37.40	93.50	38.13	95.33	37.38	93.46

Table 3. Average precision of calculated concentrations (*n = 3) expressed as % RSD, determined during three independent evaluations of fluvastatin relative response linearity in human plasma on three successive days.

Experimental concentration (ng/mL)	RSD of calculated concentrations* (%)		
	Day 1	Day 2	Day 3
0.1	15.65	15.55	17.09
0.2	6.49	9.81	5.47
0.5	1.87	3.24	2.86
1.0	2.39	3.25	2.66
5.0	4.43	2.62	4.13
10.0	5.94	4.65	3.17
25.0	4.48	3.35	1.44
50.0	1.68	4.35	2.67
0.75 (LQC)	3.92	1.88	8.42
5.0 (MQC)	3.93	0.59	0.48
40 (HQC)	2.08	1.44	2.49

Carryover may be a serious concern in bioanalytical methods, which can adversely impact the method sensitivity because the analyte response at the LLOQ level must be 10x the response seen in the blank. In addition, carryover may also affect method accuracy especially for samples containing low levels of the target analyte. Optimizing the chromatography using an Agilent flexible cube, a module in the LC stack that allows the user to backflush the needle seat, as well as enabling the needle wash after each injection, help to minimize sample retention in the autosampler. In this study, method carryover was found to be negligible. Figure 4 shows the overlay of the fluvastatin quantifier peaks in the 0.1 ng/mL LOD sample, and the first plasma blank sample injected after the upper limit of quantitation (ULOQ) sample containing 50 ng/mL fluvastatin.

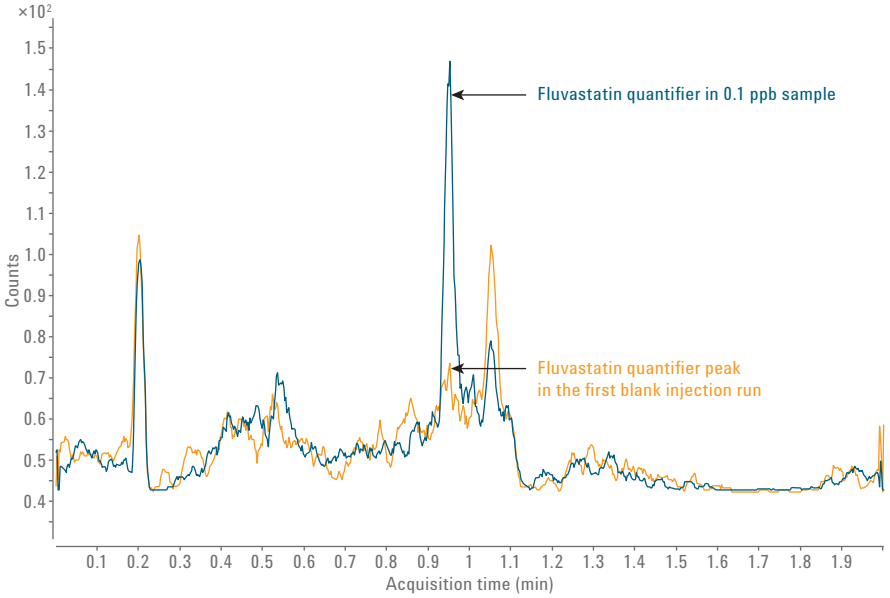

Figure 4. Overlay of fluvastatin quantifier peaks in 0.1 ppb LOD and first blank injected after the ULOQ sample.

Conclusion

A simple and rapid LC/MS/MS method for the quantification of fluvastatin in human plasma using fluvastatin-d6 as the internal standard was developed. The method has a LOD of 0.1 ng/mL, and a linear dynamic range of 0.2 to 50 ng/mL. The use of the Agilent flexible cube ensures negligible carryover of the target analyte. Good intra- and interday reproducibility, excellent precision, and accuracy at all calibration levels make the method highly suitable for routine bioanalysis of fluvastatin.

References

1. "Guidance for Industry: Bioanalytical Method Validation," U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER),Center for Veterinary Medicine (CVM), 2001. http://www.fda.gov/cder/guidance/4252fnl.htm

2. Kailasam, S. Determination of fluvastatin in plasma using the Agilent 6410B Triple Quadrupole LC/MS system coupled with the Agilent 1200 Series Rapid Resolution LC system, *Agilent Technologies Application Note*, publication number 5989-9751EN.

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