Application Note

Bioavailability/
Bioequivalence

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LC/MS/MS Method for the Low-Level Quantitation of Fluticasone Furoate in Plasma Using the Agilent 6495C Triple Quadrupole LC/MS

Figure 1. Agilent 1290 Infinity II LC coupled to an Agilent 6495C triple quadrupole LC/MS.

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Abstract

Formulated as an inhaler drug, fluticasone furoate has low bioavailability and therefore requires a sensitive bioanalytical method for low-level accurate quantification. In this application note, a selective, sensitive, and rugged bioanalytical method was developed for the accurate low-level quantification of fluticasone furoate in human plasma with an Agilent 6495C LC-TQ system. Method was found to be linear within the concentration range of 0.5 to 100 pg/mL with R^2 of 0.9971 using linear regression and $1/x^2$ weighing. QC samples at all levels showed recoveries between 90 to 115%. %RSD of area response at the lower limit of quantification (LLOQ) of 0.5 pg/mL in plasma was calculated as 5.12% with 10 replicate injections.

Introduction

Fluticasone furoate is a corticosteroid medicine used to relieve symptoms associated with asthma and allergic rhinitis. This medicine works by reducing the release of substances such as histamine that are involved in the inflamation process, thus reducing the symptoms of allergies. Fluticasone furoate is available as a nasal spray and inhaler for its low systemic bioavailability. The dose, duration, and form of fluticasone furoate depend on the condition of the patient undergoing treatment.

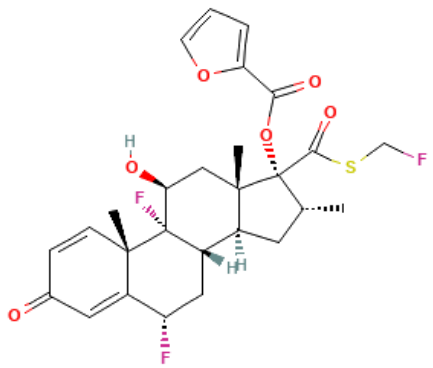


Figure 2. Structure of fluticasone furoate.

Plasma concentrations of fluticasone furoate at inhaled dose ranges are at extremely low concentrations and therefore require sensitive assays to evaluate the pharmacokinetic parameters. A highly selective and sensitive multiple reaction monitoring (MRM)-based LC/MS/MS method was developed using a 6495C triple quadrupole LC/MS (LC/TQ). The sensitivity of the 6495C LC/TQ can easily detect compounds at the required limits of detection. The special designs of the ion optics and stable electronics of the system provide consistent results across multiple batches.

Experimental

Chemicals and reagents

- Ammonium trifluoro acetate, MS grade, Sigma-Aldrich
- Formic acid, MS grade, Fluka
- Acetonitrile, MS grade, Biosolve
- Water, MS grade, Honeywell

Instrument configuration

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multisampler (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1290 Infinity II diode array detector (G7117A)
- Agilent 6495C triple quadrupole LC/MS (G6495C)

Table 1. Chromatography conditions.

Parameter	Value
Mobile Phase A	4 mM ATFA with 40 µL of formic acid in 500 mL (buffer solution)
Mobile Phase B	Acetonitrile
Flow Rate	0.8 mL/min
Injection Volume	50 µL
Sample Cooler Temp.	6 °C
Column Temperature	40 °C
Needle Wash	Acetonitrile:water (80:20)
Solvent Blank	Acetonitrile:buffer (67:33)
Column	C18 100 Å, 4.6 × 150 mm, 2.6 µm
Elution	Isocratic (38:62)
Acquisition Time	5 minutes

A standard solution of 100 ng/mL concentration was introduced to the MS by flow injection analysis with an injection volume of 5 µL. Through the automated workflow, up to 10 product ions from an analyte can be optimized for the creation of an MRM method with at least two sensitive MRM transitions.

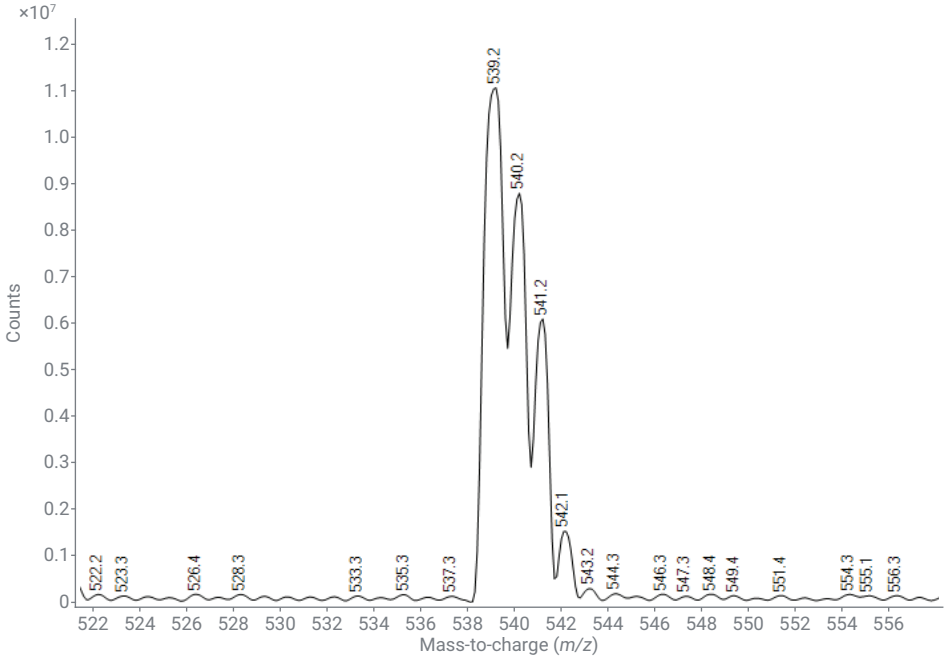
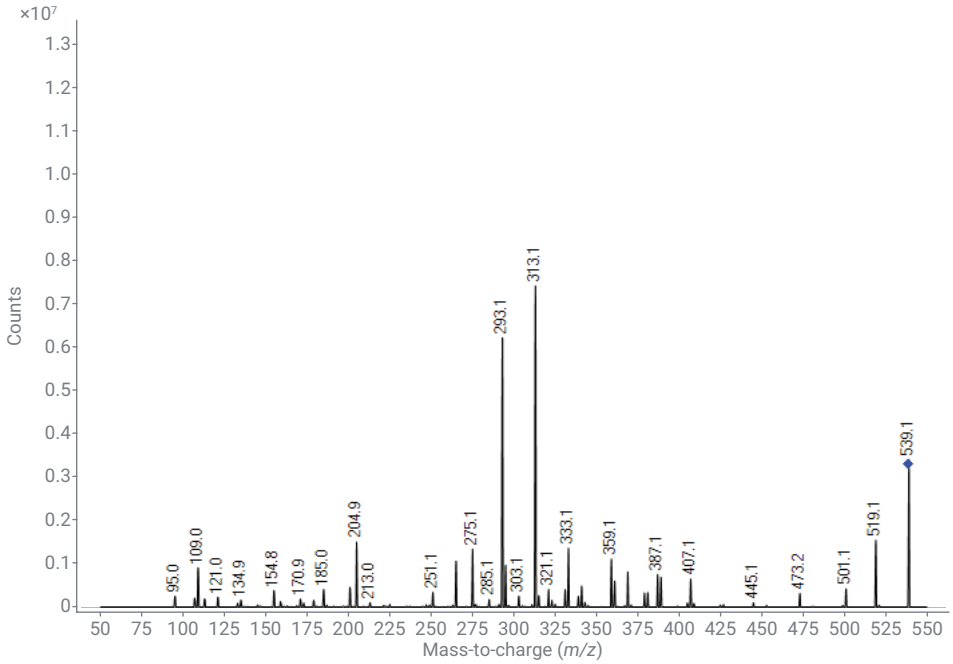


Figure 3. Q3MS scan of fluticasone furoate. The precursor ion in ESI positive mode was found to be 539.2.



Source parameters were optimized to maximize sensitivity and maintain consistency in method performance for the large precision and accuracy batches.

MRM transitions were obtained and optimized using the Agilent MassHunter Acquisition optimizer tool.

Figure 4. Fragmentation pattern of fluticasone furoate. Major fragments are *m/z* 293.1 and *m/z* 313.1.

Optimizer Report

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Instrument Information

Project Name
Instrument Name 6495
Instrument Model G6495C

Compound Name	Formula	Mass	Sample Position
FF		538.1	P1F1

Method Name
D:\MassHunter\Methods\FIA ESI POS.m

Polarity	Positive	**Ion Source**	AJS ESI

Precursor Ion	Fragmentor
539.11	166

Product Ion	Collision Energy	Abundance
293.1	16	8156051
313.1	12	9754940
275.1	28	3756886
265.1	24	2286931

Figure 5. Agilent MassHunter Optimizer Report.

Both *m/z* 313.1 and *m/z* 293.1 were selected as characteristic fragment ions, and their ion ratios were recorded throughout the batch.

Concentration levels ranging from 0.5 to 100 pg/mL were prepared as explained in Table 4. Calibration curves were plotted from 0.5 to 100 pg/mL and established the LLOQ level as 0.5 pg/mL.

Table 2. MRM parameters.

ID	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Dwell Time (ms)	Fragmentor (V)	Collision Energy (V)	Cell Accelerator Voltage (V)	Polarity
Fluticasone Furoate	538.1	293.1	80	166	16	4	Positive
	538.1	313.1	80	166	12	4	Positive
Fluticasone D3 Furoate	542.2	313.1	80	166	12	4	Positive

Table 3. MS source parameters.

Parameter	Value
Ionization Source	AJS ESI
Gas Temperature	250 °C
Gas Flow	20 L/min
Nebulizer	60 psi
Sheath Gas	400 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	4,000 V
Nozzle Voltage	300 V
High Pressure Funnel Voltage	90 V
Low Pressure Funnel Voltage	50 V

Table 4. Preparation of working standards to generate calibration curve.

Stock/QC Level	Volume of Fluticasone Furoate Stock Solutions to be Taken (µL) Into Glass Test Tube	Acetonitrile (mL)	Buffer Solution (mL)	Concentration (pg/mL)
1	200 µL of 15 pg/mL	3.80 mL	2.00 mL	0.50
2	200 µL of 45 pg/mL	3.80 mL	2.00 mL	1.50
3	200 µL of 450 pg/mL	3.80 mL	2.00 mL	15.00
4	200 µL of 900 pg/mL	3.80 mL	2.00 mL	30.00
5	200 µL of 1,200 pg/mL	3.80 mL	2.00 mL	40.00
6	200 µL of 1,800 pg/mL	3.80 mL	2.00 mL	60.00
7	200 µL of 2,400 pg/mL	3.80 mL	2.00 mL	80.00
8	200 µL of 3,000 pg/mL	3.80 mL	2.00 mL	100.00
LLOQ QC	200 µL of 15 pg/mL	3.80 mL	2.00 mL	0.50
LQC	200 µL of 45 pg/mL	3.80 mL	2.00 mL	1.50
MQC-A	200 µL of 360 pg/mL	3.80 mL	2.00 mL	12.00
MQC-B	200 µL of 1,500 pg/mL	3.80 mL	2.00 mL	50.00
HQC	200 µL of 2,400 pg/mL	3.80 mL	2.00 mL	80.00

Sample preparation

Fluticasone furoate standards were prepared in human K2EDTA plasma from 0.500 to 100 pg/mL. QC samples were prepared at 0.500, 1.5, 12, 50, and 80 pg/mL. Samples were spiked with fluticasone furoate-D_3 as internal standard solution and were subjected to protein precipitation followed by reversed-phase SPE cleanup using an C18-SPE cartridge.

Data acquisition and data analysis

All samples were acquired using Agilent MassHunter Data Acquisition software, version 10.1. Chromatograms were viewed through MassHunter qualitative analysis software, version 10.0. Quantitation of each batch was carried out using MassHunter quantitative analysis software, version 10.2. Validation parameters such as linearity, reproducibility, recovery, specificity, and sensitivity, in terms of LLOQ were characterized to ensure good method performance. Accuracies for calibration points were within ±20%. No manual integration was needed.

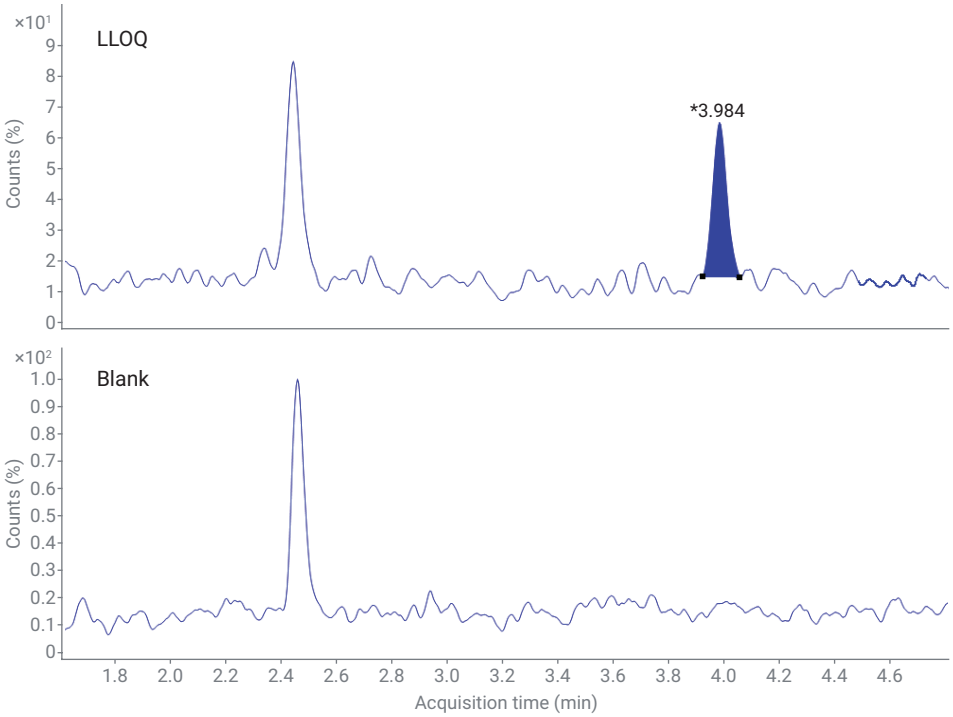

Figure 6. LLOQ chromatogram versus blank chromatogram. The retention time of fluticasone furoate is 3.98 minutes. The signal-to-noise ratio at 0.5 pg/mL in plasma was found to be 43:1, where noise is calculated by RMS.

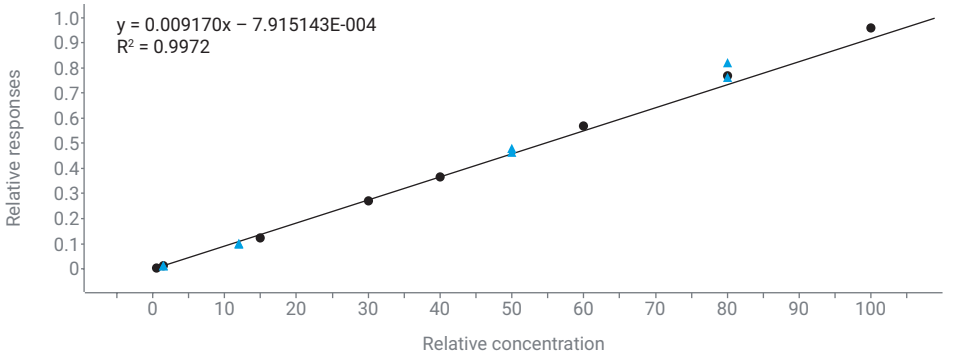

Figure 7. Calibration curves generated from 0.5 pg/mL (LLOQ) to 100 pg/mL.

Precision and accuracy batches were submitted to verify the method performance in plasma samples. A 2.5-order calibration curve generated within the concentration range of 0.5 to 100 pg/mL was found to be linear. The regression coefficient obtained was 0.9972 for linearity plotted between area ratio against concentration ratio of analyte to internal standard with a weighing factor of $1/x^2$. The accuracy of each of the calibration standards measured from the linearity curve were between 90 to 105%. Recovery for the QC samples at their respective concentrations of 1, 5, 400, and 800 ppb were between 90 to 115%.

Carryover was also evaluated by injection of the extracted blank sample after the injection of the highest standard. The % area count obtained for the blank after the injection of the highest standard was less than 5% of the area of the LLOQ sample. The signal-to-noise ratio (S/N) was calculated for LLOQ with the RMS algorithm. The S/N at LLOQ was found to be more than 40:1.

Table 5. Quantitation table showing the response and response ratio, accuracy of calibration standards, and % recovery of the QC samples.

			FF Results						FF IS (ISTD) Results	
Name	Type	Level	Exp. Conc. (pg/mL)	RT	Response	Calc. Conc. (pg/mL)	Accuracy	Ratio	RT	Response
Solvent-Blank	Double Blank	N/A	N/A	4.03	30	30.61	N/A	26.7	3.98	107
Blank Plasma 01	Blank	N/A	N/A	4	154	155.88	N/A	13.6	3.86	108
Zero Plasma 01	Blank	N/A	N/A	3.96	7	0.06	N/A	N/A	3.96	170,624
Level-01	Cal	1	0.5	3.99	827	0.51	102	70.4	3.96	213,121
Level-02	Cal	2	1.5	3.98	2,608	1.41	94	74.3	3.96	210,025
Level-03	Cal	3	15	3.98	25,655	13.58	90.5	70.3	3.96	206,972
Level-04	Cal	4	30	3.98	57,796	29.81	99.4	68.9	3.96	211,897
Level-05	Cal	5	40	3.97	71,352	40.23	100.6	67.7	3.95	193,770
Level-06	Cal	6	60	3.97	118,995	62.25	103.7	68.5	3.95	208,756
Level-07	Cal	7	80	3.97	157,595	83.82	104.8	69.8	3.95	205,265
Level-08	Cal	8	100	3.97	211,475	104.77	104.8	68.4	3.95	220,339
Blank Plasma 02	Blank	N/A	N/A	4.02	7	10.63	N/A	257.1	3.93	72
LQC-01	QC	2	1.5	3.96	2,422	1.38	92.1	73.4	3.95	199,132
MQC-A-01	QC	9	12	3.98	21,072	10.95	91.2	70	3.96	211,113
MQC-B-01	QC	10	50	3.98	89,797	51.88	103.8	69.1	3.96	189,057
HQC-01	QC	11	80	3.98	162,323	83.25	104.1	69.1	3.96	212,881
Blank Plasma 03	Blank	N/A	N/A	3.97	4	2.5	N/A	N/A	3.87	178
LQC-02	QC	2	1.5	3.98	2,411	1.39	92.9	69.2	3.95	196,343
MQC-A-02	QC	9	12	3.97	20,557	11.3	94.2	72.1	3.95	199,450
MQC-B-02	QC	10	50	3.97	92,070	50.9	101.8	71	3.95	197,577
HQC-02	QC	11	80	3.97	181,060	89.44	111.8	67.4	3.95	221,017
Blank Plasma 04	Blank	N/A	N/A	3.95	7	7.91	N/A	114.3	3.86	97

Table 6. The %RSD of 10 injections at LLOQ level.

Name	Type	Level	FF Method	FF Results		FF IS (ISTD) Results	
			Exp. Conc. (pg/mL)	RT	Response	RT	Response
Level-01	Cal	1	0.5	2.44	912	3.98	222,948
Level-01	Cal	1	0.5	2.44	911	3.98	222,487
Level-01	Cal	1	0.5	2.44	1,023	3.98	225,996
Level-01	Cal	1	0.5	2.44	1,005	3.98	226,194
Level-01	Cal	1	0.5	2.44	953	3.98	223,653
Level-01	Cal	1	0.5	2.44	1,028	3.98	214,466
Level-01	Cal	1	0.5	2.44	934	3.98	226,069
Level-01	Cal	1	0.5	2.45	1,014	3.98	224,989
Level-01	Cal	1	0.5	2.44	922	3.98	222,790
Level-01	Cal	1	0.5	2.45	1,018	3.98	219,575

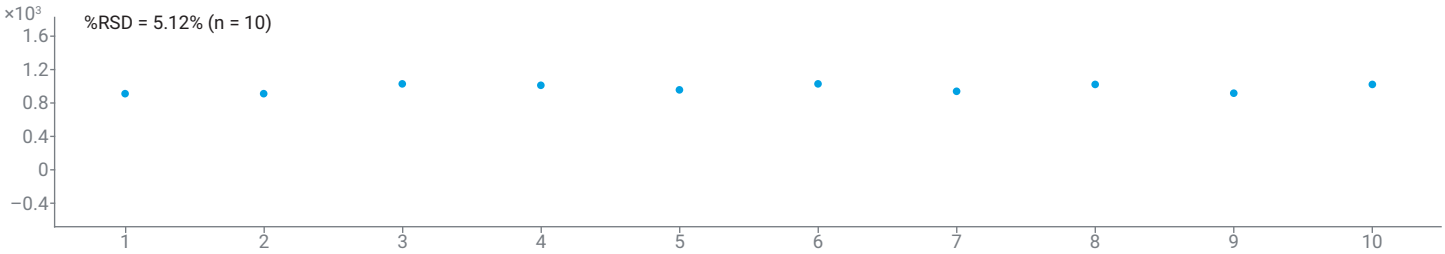

Figure 8. Metrics plot of response of 10 replicate injections at the LLOQ level, 0.5 pg/mL, in plasma showing consistency in results.

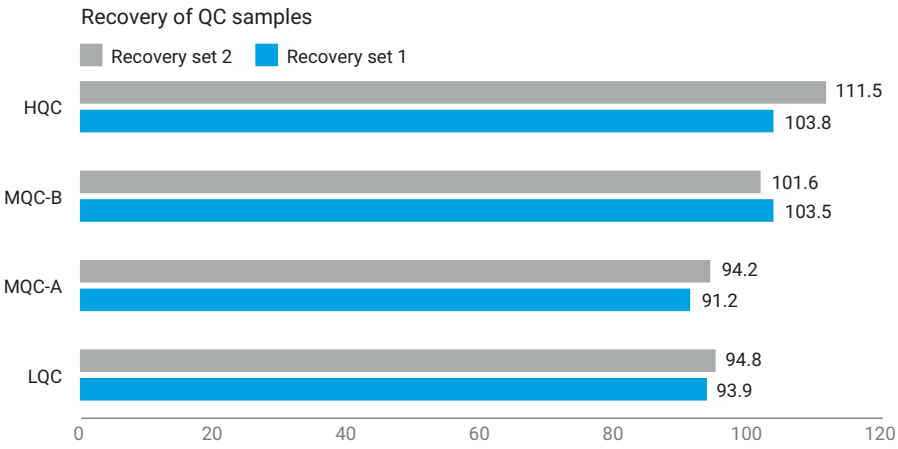

Figure 9. Bar diagram showing the recovery percentage obtained for two sets of QC samples at four different spiking levels.

Conclusion

A highly sensitive and robust MRM method was developed for the quantitation of fluticasone furoate in human plasma. The chromatographic method developed provided low matrix effect and good separation between the analyte and any interference peak. The method showed an LLOQ of 0.5 pg/mL, the lowest level in the calibration curve. The calibration curve was found to be linear within the concentration range of 0.5 to 100 pg/mL with linear regression and $1/x^2$ weighing. The R^2 value was above 0.9972. The minimum signal-to-noise ratio for fluticasone furoate at the LLOQ level was found to be more than 40:1 by RMS algorithm. Spike recovery analysis showed the efficiency of sample extraction with recovery percentage between 90 to 115% at all four spiking levels. The developed method was found to be highly reproducible at the LLOQ level, with %RSD value for the area response of 10 replicate injections not more than 7%. This method can be used to determine PK parameters in a bioequivalence study of fluticasone furoate in human plasma.

References

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RA45027.5811458333

Printed in the USA, April 25, 2023
5994-5926EN

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