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Multiple Reaction Monitoring Mass Spectrometry for the Drug Monitoring of Ivacaftor, Tezacaftor, and Elexacaftor Treatment Response in Cystic Fibrosis: A High-Throughput Method

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ABSTRACT: Ivacaftor−tezacaftor and ivacaftor−tezacaftor−elexacaftor are new breakthrough cystic fibrosis (CF) drug combinations that directly modulate the activity and trafficking of the defective CF transmembrane conductance regulator protein (CFTR) underlying the CF disease state. Currently, in the hospital setting, there are no therapeutic drug monitoring assays for these very expensive, albeit, life-saving drugs. A rapid and precise novel method for the quantification of ivacaftor, its metabolites, tezacaftor, and elexacaftor, in human plasma was developed and validated using multiple reaction monitoring mass spectrometry (MRM/MS). The MRM/MS analytical method was validated at a concentration range of 0.0025−1 μg/mL for ivacaftor, ivacaftor-M1, ivacaftor-M6, tezacaftor, and elexacaftor in human plasma. The method displayed good accuracy (90.62−94.51%) and reproducibility (99.91−100%) including at low concentrations 0.01 μg/ mL. With a mobile phase consisting of [acetonitrile/water]/0.1% formic acid (70:30 v/v) at a flow rate of 0.5 mL/min, a linear correlation was observed over a concentration range of 0.0025−1 μg/mL in human plasma for ivacaftor (R^2 = 0.9865105), ivacaftor-M1 (R^2 = 0.9852684), ivacaftor-M6 (R^2 = 0.9911764), tezacaftor (R^2 = 0.98742470), and elexacaftor (R^2 = 0.9897608). The reported method can accurately quantify ivacaftor, ivacaftor-M1, ivacaftor-M6, tezacaftor, and elexacaftor at low concentrations in human plasma. We have established a cost-efficient and timely method for measuring ivacaftor, its metabolites, and tezacaftor with or without elexacaftor in human plasma suitable for high-throughput applications in the hospital settings or clinical trials.

KEYWORDS: biomarkers, cystic fibrosis, CFTR modulators, treatment response
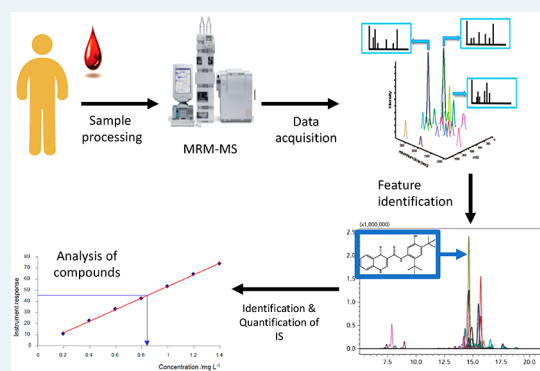
Ivacaftor−tezacaftor and ivacaftor−tezacaftor−elexacaftor combinations are the novel breakthrough cystic fibrosis (CF) treatments directly modulating the activity and trafficking of the defective CF transmembrane conductance regulator protein (CFTR).[1] CF is the most common autosomal recessive disease in Caucasian populations with serious, chronically debilitating morbidities, and high premature mortality, and at present, there is no cure.[2] Current treatment guidelines for CF recommend a regime of CFTR modulators with nonmodulator medications (e.g., anti-inflammatories or pancreatic enzymes) concomitantly administered to maintain and improve lung function, reduce the risk of infections and exacerbations, and improve quality of life. Tezacaftor and elexacaftor are second- and third-generation CFTR correctors facilitating cellular processing and trafficking of CFTR (including F508del-CFTR) to the cell surface, resulting in increase of functional CFTR protein delivered, subsequently increasing chloride transport.[3,4] Ivacaftor is a CFTR potentiator increasing the channel-open probability (or gating) of CFTR at the cell surface to increase chloride
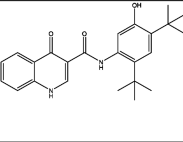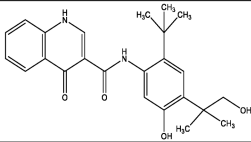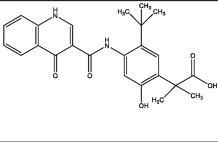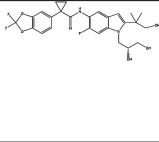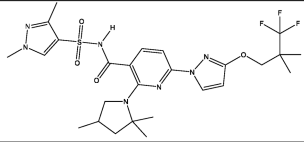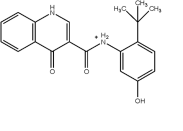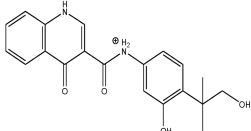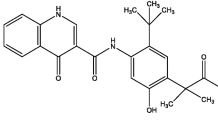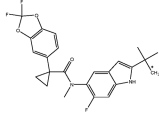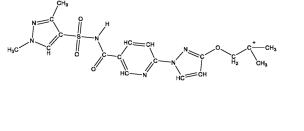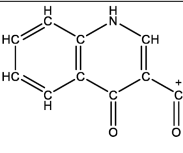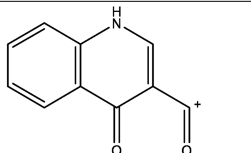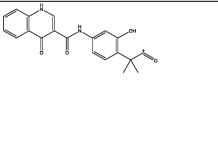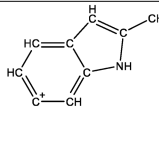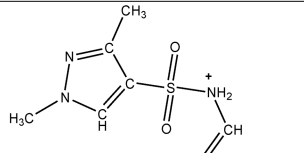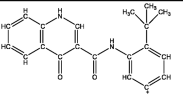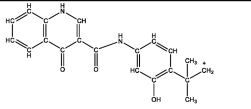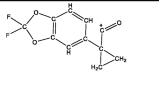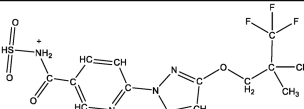
transport. Ivacaftor−tezacaftor combination was shown to be efficacious in F508del heterozygous patients carrying a CFTR residual function allele as well as displaying comparable clinical efficacy outcomes in terms of lung function [forced expiratory volume in 1 s (FEV$_1$)] and fewer side effects and drug−drug interactions than its predecessor lumacaftor.[1,5−8] Elexacaftor (VX-445) (FDA approved as of 2019, European Medicines Agency (EMA) approved as of 2020) and bamocaftor (VX-659) are next-generation CFTR correctors designed to restore F508del-CFTR protein function in patients with CF when administered with tezacaftor and ivacaftor.[9−11] Together, approximately 90% of CF patients in the United State are

Table 1. CFTR Modulators Developed for Cystic Fibrosis

Name	Ivacaftor	Ivacaftor-M1	Ivacaftor-M6	Tezacaftor	Elexacaftor
Metabolites		Ivacaftor-M1	Ivacaftor-M6		
Mode of action	Potentiator	NA	NA	Corrector	Corrector
Parent structure					
MW g·mol-1	392.49	408.9	423.1	521.1	597.7
m/z parent	393	409	423	521	598
Fragment 1					
m/z fragment 1	337	353	367	440	422
Fragment 2					
m/z fragment 2	172	172	349	130.9	205
Fragment 3			NA		
m/z fragment 3	319	335.1	NA	222.9	329

eligible for CFTR modulator therapies of either ivacaftor, N-(2,4-di*tert*-butyl-5-hydroxyphenyl)-4-oxo-1,4-dihydroquinoline-3-carboxamide $(C_{24}H_{28}N_2O_3)$, with tezacaftor, 1-(2,2-difluoro-1,3-benzodioxol-5-yl)-N-[1-[(2R)-2,3-dihydroxypropyl]-6-fluoro-2-(1-hydroxy-2-methylpropan-2-yl)indol-5-yl]-cyclopropane-1-carboxamide $(C_{26}H_{27}F_3N_2O_6)$, or ivacaftor–tezacaftor with elexacaftor, (S)-N-((1,3-dimethyl-1H-pyrazol-4-yl)sulfonyl)-6-(3-(3,3,3-trifluoro-2,2-dimethylpropoxy)-1H-pyrazol-1-yl)-2-(2,2,4-trimethylpyrrolidin-1-yl)nicotinamide $(C_{26}H_{34}F_3N_7O_4S)$ combination (Table 1).[12]

Ivacaftor–tezacaftor is administered orally with the recommended dose being one tablet (100 mg of tezacaftor+150 mg of ivacaftor) taken in the morning and one tablet of ivacaftor (150 mg only), taken in the evening, approximately 12 h apart.[1] Ivacaftor–tezacaftor–elexacaftor combination is given orally with the recommended dose is one tablet (200 mg of elexacaftor+100 mg of tezacaftor+150 mg of ivacaftor) taken in the morning and one tablet of ivacaftor (150 mg) only, taken in the evening. Similar to ivacaftor monotherapy and ivacaftor–lumacaftor (first-generation CFTR potentiator), ivacaftor–tezacaftor are taken with high-fat-containing food to increase bioavailability. Following its oral administration, both ivacaftor, tezacaftor, and elexacaftor are readily absorbed from the gut; however, they have low solubility in water (<0.05 μg/mL). Tezacaftor and ivacaftor exhibit a T_{max} of ~4 and ~6 h, respectively.[13] For the triple combination T_{max} is as follows: 6 h (4−12) elexacaftor, 3 h (2−4) tezacaftor, and 4 h (3−6)

ivacaftor. If given alone, then the half-life of ivacaftor is 12−14 h, while the half-life of tezacaftor is 111 h (Table 2). The half-life of the combination is changed to 9.3 h for ivacaftor and 156 h for tezacaftor.[14] The annual cost per patient for both drugs is high, and presently, their manufacturer has a market monopoly as these are the only drugs on the market available for their indication. Presently, very little information is available concerning the steady-state plasma concentrations of ivacaftor–tezacaftor and ivacaftor–tezacaftor–elexacaftor combinations achievable in patients under the current recommended dosage regimen; no methods for therapeutic drug monitoring have been reported so far. We have previously reported an analytical method for the quantification of ivacaftor–lumacaftor treatment.[15,16] The application of the ivacaftor–lumacaftor analytical assay was implemented in the hospital setting and utilized for a number of clinical studies.[17−20] With the clinical shift from ivacaftor–lumacaftor to ivacaftor–tezacaftor and ivacaftor–tezacaftor–elexacaftor therapy, we sought to adapt and optimize our previous HPLC/LC-MS to the changing landscape in CF standard care.[10,15,16] The objective of this study was to develop a reliable and simple multiple reaction monitoring mass spectrometry (MRM-MS) method for the quantification of ivacaftor, its major metabolites ivacaftor-M1 and ivacaftor-M6, tezacaftor, and elexacaftor in plasma for therapeutic drug monitoring of CF patients receiving ivacaftor–tezacaftor therapy. MRM assays are directed tandem mass spectrometric techniques performed

Table 2. ADME Profile of Ivacaftor and Ivacaftor–Tezacaftor and Ivacaftor–Tezacaftor–Elexacaftor Standard Therapy[a]

	ivacaftor	tezacaftor	elexacaftor	ivacaftor+tezacaftor	ivacaftor+tezacaftor+elexacaftor
			Pharmacokinetics		
description	film-coated tablet: 150 mg	100 mg once daily (clinical trial)	elexacaftor, 200 mg once daily	tezacaftor, 100 mg once daily + ivacaftor, 150 mg every 12 h	elexacaftor, 200 mg and tezacaftor, 100 mg, once daily + ivacaftor 150 mg, every 12 h
mean AUC	5260 ng·hr/mL	57400 ng·hr/mL		82.7 μg·h/mL tezacaftor; 10.9 μg·h/mL ivacaftor	162 (48.1) μg·h/mL elexacaftor; 94.5 (24.0) μg·h/mL tezacaftor; 11.7 (4.01) μg·h/mL ivacaftor
mean C_{max}	233 ng/mL	5530 ng·hr/mL		6.52 μg/mL tezacaftor and 1.28 μg/mL ivacaftor	8.7 (2.1) μg/mL elexacaftor; 6.8 (1.5) μg/mL tezacaftor; 1.2 (0.3) μg/mL ivacaftor
steady state reached	3–5 days with accumulation ratio of 2.2–2.9	within 8 days with accumulation ratio of 1.5	within 14 days with accumulation ratio of 2.3	at steady state: accumulation ratio = 2.3 for tezacaftor and 3.0 for ivacaftor	at steady state: accumulation ratio = 3.6 for elexacaftor, 2.8 for tezacaftor, and 4.7 for ivacaftor
			Absorption		
half-life	12–14 h	111 h		156 (52.7) h tezacaftor; 9.3 (1.7) h ivacaftor	6 h (4–12) elexacaftor, 3 h (2–4) tezacaftor, 4 h (3–6) ivacaftor
increase in exposure	from 25 mg every 12 h to 450 mg every 12 h	Tezacaftor exposure increased from 10 mg to 300 mg once daily when administered alone or in combination with ivacaftor.		Tezacaftor exposure increased from 10 mg to 300 mg once daily when administered alone or in combination with ivacaftor.	20% increase in ivacaftor exposure in the presence of tezacaftor
increase in exposure due to food effect	increased 2–4 fold if given with fat containing food	No relevant food effect on exposure to tezacaftor	AUC increases 1.9–2.5 fold with moderate-fat meal		
			Distribution		
median (range) t_{max}	4.0 (3.0–6.0) h	3.0 (2.0–4.0) h	6.0 (4.0–12.0) h	4.0 (2.0–8.0) h tezacaftor; 6.0 (3.0–10.0) h ivacaftor	6 (4–12) h elexacaftor; 3 (2–4) h tezacaftor; 4 (3–6) h ivacaftor
plasma proteins	99% bound to HSA and AGP	99% bound to HSA	>99% bound to HSA		
volume of distribution	293 (89.8) L	82.0 (22.3) L	53.7 (17.7) L	271 ± 157 L tezacaftor; 206 ± 82.9 L ivacaftor	53.7 (17.7) L elexacaftor; 82.0 (22.3) L tezacaftor; 293 (89.8) L ivacaftor
			Metabolism		
cytochrome metabolism	CYP3A4	CYP3A4/5	CYP3A4/5		
metabolites	ivacaftor-M1 (partially active), ivacaftor-M6 (inactive)	tezacaftor-M1 (active), tezacaftor-M2 (partially active), tezacaftor-M5 (inactive)	M23-ELX (active)		
			Excretion		
elimination in faeces	88% (22% as M1 and 43% as M6)	72% (unchanged or as tezacaftor-M2)	87.3% (primarily as metabolites)		
urinary elimination	6.6% (ivacaftor, M1 and M6)	14% mostly tezacaftor-M2	0.23%		
terminal half-life	elimination half-life: 9.3 (1.72) h	elimination half-life: 109–122 h			24.7 (4.87) h elexacaftor; 60.3 (15.7) h tezacaftor; 13.1 (2.98) h ivacaftor
apparent clearance (CL/F)	17.3 (±8.4) L/h at steady state	total systemic clearance 1.34 L/h		1.31 (±0.41) L/h tezacaftor; 15.7 (±6.38) L/h ivacaftor	1.18 (0.29) L/h elexacaftor; 0.79 (0.10) L/h tezacaftor

Table 2. continued

ivacaftor	tezacaftor	elexacaftor	ivacaftor+tezacaftor	ivacaftor+tezacaftor+elexacaftor
		Distribution		10.2 (3.13) L/h ivacaftor

[a]ADME = absorption, distribution, metabolism and excretion. Adapted from Hanafin et al.[17,28]

onto triple quadrupole mass spectrometers for the sensitive and specific analysis of compounds and their metabolites in biological samples.[21−24] The implementation of the assay is of great clinical utility for pharmacokinetic monitoring and developing exposure−response relationships to maximize drug efficacy.

MATERIALS AND METHODS

Materials. Ivacaftor, tezacaftor, and elexacaftor were purchased from SelleckChem, USA. Ivacaftor−carboxylate/ivacaftor-M6 metabolite (catalog number 510242247CS) and hydroxymethyl−ivacaftor/ivacaftor-M1 metabolite (catalog number 510240849CS) were purchased from Clearsynth (Canada) and were used as internal standards (IS) in methanol at 10 μg/mL. Methanol (MeOH, ≥99.9% HPLC LC-MS grade), acetonitrile (≥99.9% HPLC LC-MS grade), and LC-MS-grade formic acid were purchased from Sigma-Aldrich (Australia). Experiments were performed on a triple-quadrupole Shimadzu 8030 LC-MS using a C8 column (Phenomenex; 2.6 μm, 100 Å; 50 × 2.1 mm) with the guard column C8 (Phenomenex; 2.1 mm ID Columns, 3/Pk). Plasma was obtained from the Australian Red Cross.

Standards Preparation. Independent stock solutions of internal standard of each analyte (ivacaftor, ivacaftor-M1, ivacaftor-M6, tezacaftor, and elexacaftor) were freshly prepared in methanol for each analytical run at 100 μg/mL and by further dilutions working stock solutions of 0.8 μg/mL were prepared. Ten calibration standards stock solutions of each analyte at 0.001, 0.0025, 0.005, 0.01, 0.025, 0.05, 0.1, 0.025, 0.5, and 1 μg/mL were prepared in methanol by serial dilution of the 100 or 0.8 μg/mL stock solutions.

Plasma Sample Processing. Blank plasma samples are spiked with international standards at the concentration range described above and vortex-mixed before sampling, and an aliquot of 200 μL was transferred into a 1.5 mL polypropylene microcentrifuge tube. Protein precipitation was achieved through a 1:2 precipitation using a mixture of acetonitrile/0.1% formic acid. The mixture was set at room temperature for 10 min, then vortexed vigorously, then centrifuged at 10 000 × g for another 10 min (Eppendorf Centrifuge 5430). A 200 μL aliquot of the clear supernatant was filtered through a 13 mm syringe filter (0.45 μm nylon, GRACE, USA) into a 1.5 mL HPLC vial [Phenomenex VEREX, 9 mm, PP, 300 μL, PTFE/Silicone septa]. An aliquot of 5 μL was injected onto the column.

MRM Method Development. In an MRM assay, the sensitivity of an MRM ion pair is directly related to the signal intensity of the fragment ion that is transmitted through quadrupole 3 (Q3) and allowed to strike the detector.[21] Briefly, all analytes (0.8 μg/mL in methanol and the corresponding diluted standards) alone were injected in positive and negative mode to determine which Q3 mode generates the highest signal. In a second step, the collision energy voltage ranging from in the mass spectrometer were varied (−10 to −40 V), which affects the fragmentation pattern of analytes and the relative intensities of the fragment ions. Third, fragment precursors were calculated based on predicted m/z values and identified for each analyte. For each MRM ion pair, the extracted ion chromatograms are generated, and the five highest intensity peaks identified based on their corresponding collision energy (CE). Finally, the optimized MRM acquisition method is generated. Details are found in Table 3.

Table 3. Analyte Parameters for Ivacaftor, Ivacaftor-M1, Ivacaftor-M6, Tezacaftor, and Elexacaftor

compound	precursor *m/z*	product *m/z*	+/−	dwell time (msec)	Q1 preBias (V)	CE	Q3 preBias (V)
		337.0	+	10.00	−19.0	−15.0	−17.0
ivacaftor	393.1	172.0	+	10.00	−11.0	−50.0	−21.0
		319.0	+	10.00	−11.0	−49.0	−29.0
		353.0	+	10.00	−30.0	−17.0	−28.0
ivacaftor-M1	408.5	172.0	+	10.00	−10.0	−55.0	−20.0
		335.1	+	10.00	−12.0	−55.0	−30.0
		367.0	+	10.00	−17.0	−14.0	−28.0
ivacaftor-M6	423.1	346	+	10.00	−15.0	−35.0	−15.0
		440.0	+	10.00	−26.0	−19.0	−24.0
tezacaftor	521.1	130.9	+	10.00	−24.0	−53.0	−15.0
		222.9	+	10.00	−22.0	−42.0	−22.0
		422	+	10.00	−26.0	−55.0	−18.0
elexacaftor	598	205	+	10.00	−36.0	−55.0	−29.0
		329	+	10.00	−26.0	−54.0	−20.0

MRM-MS Analytics. The analysis of plasma samples was performed on a Shimadzu 8030 LCMS system coupled with the 8030 triple quadrupole mass spectrometers. Details on the MRM-MS conditions are summarized in Table 4. The mobile phase flow was split before entering the mass spectrometer in ratio 2:1 (waste/MS inlet). Analytes were detected using multiple reaction monitoring (MRM). The ion transitions of ivacaftor 393.1 → 337.0 *m/z*, ivacaftor-M1 408.5 → 353.0 *m/z*, ivacaftor-M6 423.1 → 367.0 *m/z*, tezacaftor 521.1 → 440.0 *m/z*, and elexacaftor 597.7 → 598 *m/z*. Chromatographic separation was achieved using a C8 column (2.6 μm; 100 Å; 50 × 2.1 mm) equipped with a guard column C8 (2.1 mm ID Columns, 3/Pk). The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B). Injection volume was 2 μL. The gradient started at 30% B at time 0 and increased to 90% B by 4.00 min, kept at 90% B until 4.5 min, and then reduced to 20% B by 5.50 min. After each injection, the needle was washed with 300 μL of 80% methanol, 20% water, and gradient then returned to 30% B.

Method Validation. The method validation procedure for ivacaftor−tezacaftor and ivacaftor−tezacaftor−elexacaftor were based on the FDA guidelines for bioanalytical method validation.[25] Partially method validation was performed including parameters listed in Table 5 as the aim was to develop a method that is easily accessible for diagnostic laboratories in a clinical setting measuring all four compounds in one single assay.

For the level 1 analysis, MRM data processing and analyte peak recognition was achieved using integration software postrun (Shimazu). Default peak integration settings were set to detect peaks using a 1 min retention window around the analytes' retention time. Default values were subtracted for noise and baseline. All integrated chromatograms were manually inspected (level 2 analysis) to ensure the software recognized and integrated the correct peak for each MRM ion pair. The calibration curves for ivacaftor, ivacaftor-M1, ivacaftor-M6, tezacaftor, and elexacaftor were constructed before each analytical run using the relationship between the peak area ratios of analyte/fragment to international standards and the calibration standard nominal concentrations of ivacaftor, ivacaftor-M1, ivacaftor-M6, tezacaftor, and elexacaftor (Figure 1). Linear least-squares regression analysis with weighting $1/C^2$ was performed according to the reciprocal of concentrations (Shimadzu).

Table 4. Details of the LC-MS/MS Conditions

HPLC Conditions	
column	C8; 2.6 μm; 100 Å; 50 × 2.1 mm
guard column	C8 for 2.1 mm I.D. columns, 3/Pk
oven temperature	35 °C
mobile phase A	0.1% FA in water
mobile phase B	100% acetonitrile
mode	binary gradient
mobile phase composition at starting point	70/30 (v/v)
sample temperature	4 °C
injection volume	2 μL
needle wash	80% methanol, 20% water 300 μL
flow rate	0.5 mL/min
gradient	time 0: 30% B
	from 0 to 4.00 min: kept at 30% B
	by 4.00 min: 90% B
	from 4.00 to 4.50 min: kept at 90% B
	from 5.00 min: 30% B
	from 5.00 to 5.50 min: kept at 30% B
total run	5.5 min
retention times	retention time for ivacaftor: 2.248 min
	retention time for ivacaftor-M1: 1.136 min
	retention time for ivacaftor-M6: 0.981 min
	retention time for tezacaftor: 1.719 min
	retention time for elexacaftor: 3.306 min
MS Conditions	
detection mode	electrospray positive ESI
interface voltage	4.5 kV
interface current	1.9 uA
collision energy	295.9 V
nebulizing gas (nitrogen)	3 L/min
drying gas (nitrogen) flow	15 L/min
desolvation temperature	250 °C
heat block temperature	400 °C
conversion dyno	6.0 kV
detector voltage	2.18 kV
IG vacuum	1.9×10^{03} Pa
PG vacuum	$1.1 \times 10^{+02}$ Pa
CID gas	230 kPa

Table 5. Ivacaftor, Ivacaftor Metabolites, Tezacaftor and Elexacaftor Analytical Parameters Including LOD, LOQ, Recovery, and Inter- and Intraday Precision and Accuracy

		ivacaftor [μg/mL]	ivacaftor-M1 [μg/mL]	ivacaftor-M6 [μg/mL]	tezacaftor [μg/mL]	elexacaftor [μg/mL]
LOD		8.46×10^{-03}	2.54×10^{-02}	2.27×10^{-02}	1.80×10^{-02}	7.40×10^{-03}
LOQ		2.82×10^{-02}	8.46×10^{-02}	7.58×10^{-02}	6.00×10^{-02}	2.47×10^{-02}
accuracy (%)		91.87 ± 0.007	94.51 ± 0.04	92.97 ± 0.03	90.62 ± 0.04	92.27 ± 0.09
interday	0.1 μg/mL	100.0 ± 0.16	96.22 ± 5.28	91.95 ± 9.98	94.36 ± 6.61	96.55 ± 4.89
	1.0 μg/mL	99.91 ± 0.11	100.45 ± 0.62	100.48 ± 0.67	100.46 ± 0.67	100.44 ± 0.61
intraday	0.1 μg/mL	98.00 ± 0.36	80.00 ± 0.38	92.50 ± 0.44	95.50 ± 0.46	95.33 ± 0.17
	1.0 μg/mL	95.45 ± 0.06	93.90 ± 0.03	97.90 ± 0.01	95.20 ± 0.06	98.05 ± 0.01
recovery from plasma (%)		78.21 ± 5.44	84.2 ± 18.2[15]	101.52 ± 2.14	94.22 ± 1.46	111.93 ± 8.80

[a]Abbreviations: lower limit of detection (LOD), lower limit of quantification (LOQ)
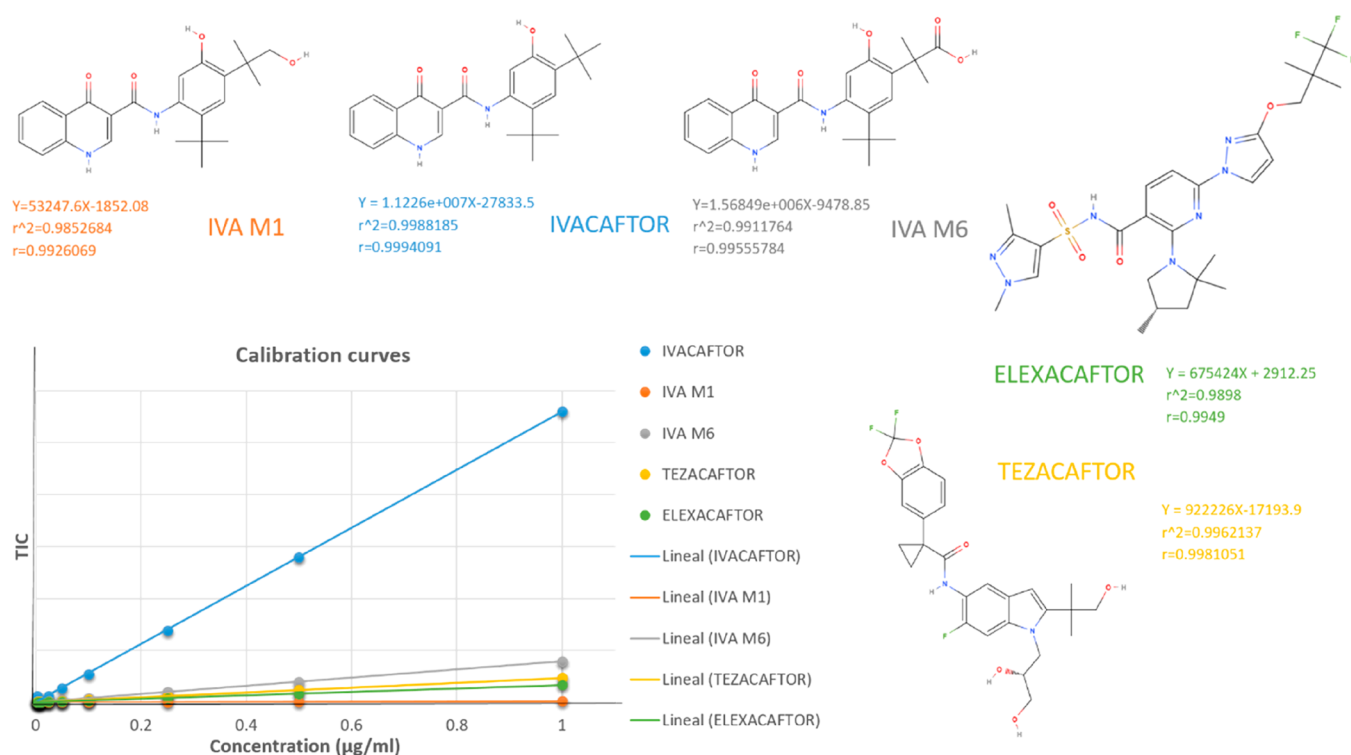
Figure 1. Calibration curves of ivacaftor, ivacaftor-M1, ivacaftor-M6, and tezacaftor.

■ RESULTS

Method Development. We have previously reported reliable HPLC/LC-MS methods for the detection and quantification of ivacaftor and lumacaftor.[15,16] The MRM-MS method herein was based on the chromatographic separation and peak shape of blank plasma spiked with standards ivacaftor, ivacaftor-M1, ivacaftor-M6, tezacaftor, and elexacaftor. Analyte specific transition settings such as precursor *m/z*, Q1 pre-rod bias voltage, product *m/z*, collision energy, and Q3 pre-rod bias (Tables 3 and 4) were optimized using LabSolutions software (Shimadzu, Japan 2019) protocols from flow injections analysis of mixed pure standards containing all four analytes at a concentration of 0.1 μg/mL. HESI source parameters were manually optimized. Baseline peak separation for ivacaftor, ivacaftor-M1, ivacaftor-M6, tezacaftor, and elexacaftor was achieved using reverse-phase chromatography. As shown in Table 3, in source fragmentation of analytes resulted in the following specific transitions ranging from highest to lowest intensity, ivacaftor 393 > 337 > 172 > 319 *m/z*, ivacaftor-M1 408.5 > 353 > 172 > 335.1 *m/z*,

ivacaftor-M6 423 > 367 > 349 *m/z*, tezacaftor 521.1 > 440 > 130.9 > 222.9 *m/z*, and elexacaftor 597.7 > 422 > 205 > 329 *m/z*, being produced at the following retention times, ivacaftor 2.25 min, ivacaftor-M1 1.14 min, ivacaftor-M6 0.98 min, tezacaftor 1.72 min, and elexacaftor 3.31 min. Furthermore, the following unspecific transitions were detected; however due to their nonspecificity not used for peak quantifications: ivacaftor 302 > 209 > 57 *m/z*, ivacaftor-M1 281 > 164 *m/z*, ivacaftor-M6 311 > 291 > 209 > 126 > 11 *m/z*, tezacaftor 449 > 420 > 291 > 131 *m/z*, and elexacaftor 502 *m/z* (Figure 2). Thus, good peak separation is essential to avoid signal contamination. In all spiked plasma samples, no interference with the retention time of either of the analytes was observed, nor was interference detected from the combination of ivacaftor, ivacaftor-M1, ivacaftor-M6, tezacaftor, and elexacaftor. After each injection the LC column was washed for another 2.5 min with 90% acetonitrile and the equilibrated to starting conditions (20% acetonitrile). The total running time per sample is 5.5 min, which is of great interest for diagnostic centers analyzing large amounts of samples.
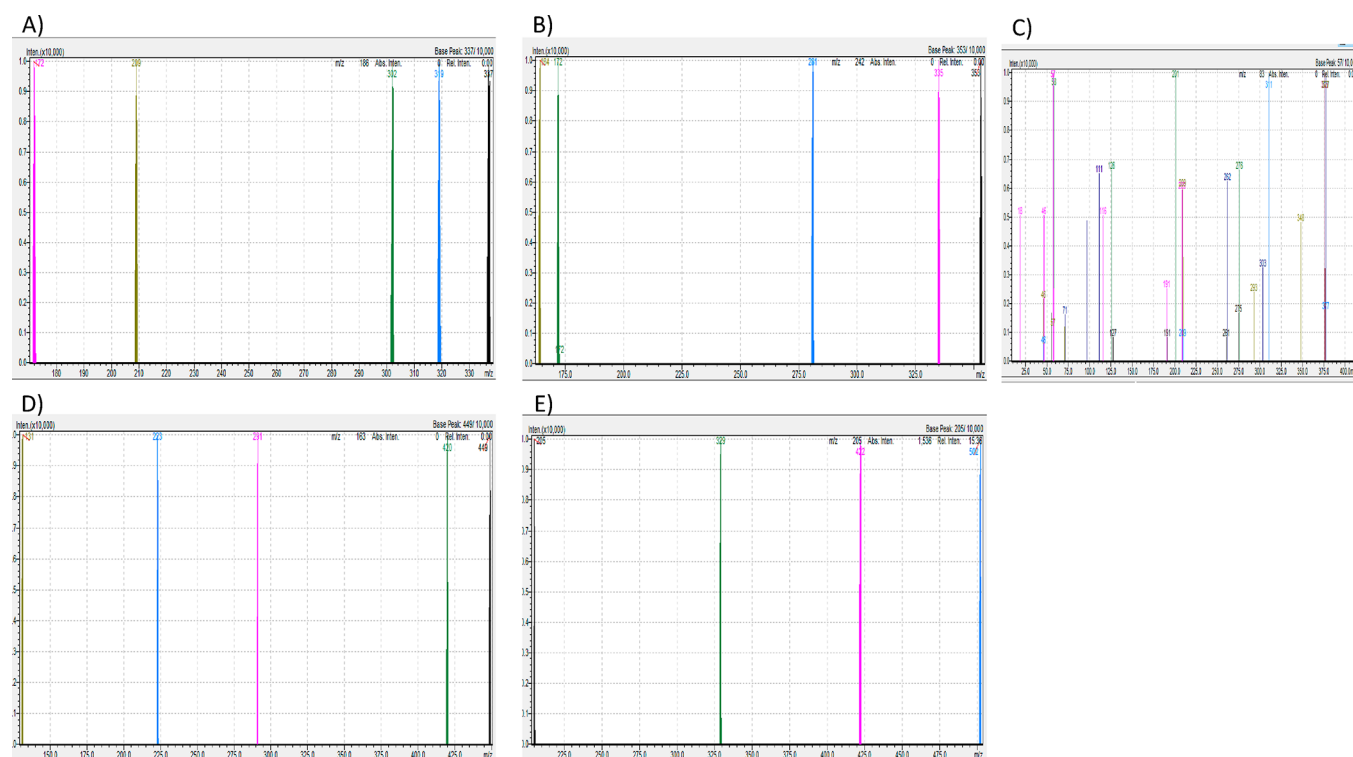
Figure 2. MRM spectra of fragmentation behavior of (A) ivacaftor, (B) ivacaftor-M1, (C) ivacaftor-M6, (D) tezacaftor, and (E) elexacaftor.

Sample Preparation. In our previous HPLC/LC-MS techniques we have reported a 1:1 preparation involving protein precipitation sample/[mixture of acetonitrile/0.1% formic acid].[15] Given the large number of samples used in our studies, we observed protein precipitation after sample injection (data not shown). We aimed to eliminate unnecessary and time-consuming clean up steps by first precipitating proteins at a 1:2 ratio (sample/[mixture of acetonitrile/0.1% formic acid]) and second by starting the gradient at 70% acetonitrile (rather than 60% as per our previous method).

Accuracy and Linearity. As shown in Table 5 good accuracy (90.62−94.51%) was observed within the linear range of 0.0025−1 μg/mL for ivacaftor, ivacaftor-M1, ivacaftor-M6, tezacaftor, and elexacaftor and using area ratio in the respective calibration curves (Figure 2). Linear fit with $1/A^2$ weighting factor was employed for all four target components. Good fit to these four models were observed as represented by the correlation coefficients ($R^2 = 0.98-0.99$). As shown in Figure 2 and Table 5, good reproducibility (interday 99.91−100%) was observed within the linear range of 0.0025−1 μg/mL for ivacaftor, ivacaftor-M1, ivacaftor-M6, tezacaftor, and elexacaftor. Accuracy and precision were calculated via relative standard deviation (RSD). For each quality control, the RSD values should be less than 15%.[26] For this analytical method, a $1/C^2$ linear equation as the weighing factor was chosen to achieve a linear regression; the linear equations were as follows: ivacaftor ($y = 5.82353 \times 10^{+006}X - 121\,332$; $R^2 = 0.9865105$; $r = 0.9932323$), ivacaftor-M1 ($y = 53\,247.6X - 1852.08$; $R^2 = 0.9852684$; $r = 0.9926069$), ivacaftor-M6 ($y = 1.56849 \times 10^{+006} - 9\,478.85$; $R^2 = 0.9911764$; $r = 0.9955784$), tezacaftor ($y = 1.63653 \times 10^{006}x - 53\,488.3$; $R^2 = 0.98742470$; $r = 0.9936936$), and elexacaftor ($y = 922\,226X - 7193.9$; $R^2 = 0.9962137$; $r = 0.9981051$). Good fit to this model was

observed as represented by the correlation coefficient $R^2 > 0.98-0.99$.

Limit of Quantification. In all blank plasma samples, no interference with the retention time of either of the analytes was observed, nor was interference detected from the combination of ivacaftor with tezacaftor with or without elexacaftor. The method used to determine the endogenous interferences was the percentage of the area of the lower limit of detection (LOD) for each analyte. The LOD and quantification (LOQ) were established: for ivacaftor, LOD 8.46×10^{-03} μg/mL and LOQ 2.82×10^{-02} μg/mL; for ivacaftor-M1, LOD 2.54×10^{-02} μg/mL and LOQ 8.46×10^{-02} μg/mL; for ivacaftor-M6, LOD 2.27×10^{-02} μg/mL and LOQ 7.58×10^{-02} μg/mL; for tezacaftor, LOD 1.80×10^{-02} μg/mL and LOQ 6.00×10^{-02} μg/mL; and for elexacaftor, LOD 7.40×10^{-03} μg/mL and LOQ 2.47×10^{-02} μg/mL (Table 5).

Precision. In this study, the precision of our analytical procedure was expressed as RSD or coefficient of variation of a series of measurements. The relative standard deviation was calculated by taking the standard deviation of the sample set multiplied by 100% and dividing it by the sample set average. The relative standard deviation is expressed as percent:

$$\%RSD = s/x \times 100\%$$

with s being the standard deviation and x the average of the three independent measurements.

To confirm the precision, the predicted %RSD has to be compared with the experimental %RSD, as follows:

- % RSD theoretical = 2 exp (1−0.5 log C), with C being the real concentration of the sample

- Good interday precision: %RSD exp $\leq$ %RSD theoretical

- Good intraday precision: %RSD exp $\leq$ %RSD theoretical/2

Intraday repeatability was below 1% RSD for all four compounds ivacaftor, ivacaftor-M1, ivacaftor-M6, and tezacaftor at 1 μg/mL (quality control level, n = 6). Interday variability was determined on 3 different days and was below 8.16%RSD for ivacaftor and tezacaftor at low concentrations (0.1 μg/mL) and below 5.4%RSD at 1 μg/mL (Table 5), indicating good precision.

Recovery from Plasma. The combination of acetonitrile/ 0.1% formic acid provided optimal protein precipitation and excellent recovery of all five analytes from the plasma matrix. Recovery was 78.21% for ivacaftor, 84.20% for ivacaftor-M1, 101.52% for ivacaftor-M6, 94.22% for tezacaftor, and 111.93% for elexacaftor, respectively, indicating that all five analytes could be quantitatively recovered from plasma. The relative recovery of ivacaftor, ivacaftor-M1, ivacaftor-M6, and tezacaftor was calculated by comparing the peak areas of the samples prepared in human plasma with those obtained from a set of quality control samples prepared in methanol. High recoveries of all five analytes were observed and are in line with our previously reported recoveries for ivacaftor and its metabolites (Table 5).

DISCUSSION

Herein, we describe a rapid, simple, and sensitive methodology to allow the high-throughput MRM-MS analysis of plasma samples measuring ivacaftor, ivacaftor-M1, ivacaftor-M6, tezacaftor, and elexacaftor concentrations in CF patients receiving ivacaftor−tezacaftor or ivacaftor−tezacaftor−elexacaftor combination therapy. The analysis time is reduced to 5.5 min, and the sample preparation is straightforward and time efficient. These methodological changes allow a state-of-the-art use for the changing landscape in CF therapy as well as processing a large number of samples which are used in hospital setting or required for clinical trials. MRM-MS methods have in the past been used as a biomarker tool in the context of CF diagnostics. Roberts et al. have used MRM-MS to identify novel plasma protein biomarkers in response to CF lung exacerbations.[24] Quon et al. have used MRM-MS to predict imminent CF pulmonary exacerbations by quantifying novel plasma protein biomarkers.[23]

We have previously reported analytical methods for the quantification of ivacaftor−lumacaftor patient samples; however, with standard treatment regimens shifting, we must adapt and optimize the reported methods. Although Singh et al. have recently reported a HPLC method for the quantification of tezacaftor and ivacaftor, our method herein allows for a faster and low-dose high-throughput analysis with the inclusion of important active and partially metabolites as well as the inclusion of the novel triple combination.[27] Targeted method MRM-MS analysis such as the method described herein requires knowledge of the molecular weight of the analytes and their fragmentation behavior: ivacaftor 393 > 337 > 172 > 319 m/z, ivacaftor-M1 408.5 > 353 > 172 > 335.1 m/z, ivacaftor-M6 423 > 367 > 349 m/z, tezacaftor 521.1 > 440 > 130.9 > 222.9 m/z and elexacaftor 597.7 > 422 > 205 > 329 m/z under collision-induced dissociation (CID) conditions (Tables 3 and 4). By combining carefully selected MRM precursor and fragment ion pairs, MRM can be used to determine, highly specifically and reproducibly, the absolute concentrations of ivacaftor and its metabolites, tezacaftor, and elexacaftor in

biological samples. The concentrations of the analyte fragments can be used to infer the concentrations of the precursor compounds.[21]

In summary, we have demonstrated for the first time that MRM-MS can be applied as a biomarker tool to quantify concentrations of ivacaftor and tezacaftor and elexacaftor in CF. MRM-MS is a platform that has been used in clinical diagnostic laboratories to measure drugs and their metabolites, but to date no assays have been reported for the monitoring of ivacaftor−tezacaftor combination. Given that drug monitoring may be essential in certain cohorts, e.g., CF patients with severe liver impairment, pregnant women, or poor responders to CFTR modulator treatment, therapeutic drug monitoring in patient plasma is requisite to achieve optimizing dosage regimens and allow pharmacokinetic/pharmacodynamic analyses. The deployment of this simple yet sensitive assay in the hospital setting will offer clinicians the rationale to develop exposure−response relationships, to aid with the development and understanding of predictive biomarkers, and to achieve the best possible clinical efficacy. Further research will include clinical validation and evaluation for a clinical utility study.

CONCLUSION

This rapid and sensitive methodology allows for the high-throughput MRM-MS analysis of plasma samples from patient with CF receiving ivacaftor−tezacaftor or ivacaftor−tezacaftor−elexacaftor combination therapy. Our methodological adaptions allow for a large number of patient samples which are required for clinical trials including dose-finding studies, pharmacokinetic/pharmacodynamic analysis, or the routine hospital screening. A simple and minimally invasive plasma test can be applied to mitigate potential drug interactions.

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F.R.-O., F.Q., and E.K.S.-F. were involved in the conception and design, analysis and interpretation of the data and the drafting of the paper or revising it critically for intellectual content and are responsible for the final approval of the version to be published. All authors agree to be accountable for all aspects of the work.

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