Video Article

Optimized LC-MS/MS Method for the High-throughput Analysis of Clinical Samples of Ivacaftor, Its Major Metabolites, and Lumacaftor in Biological Fluids of Cystic Fibrosis Patients

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Abstract

Defects in the cystic fibrosis trans-membrane conductance regulator (CFTR) are the cause of cystic fibrosis (CF), a disease with life-threatening pulmonary manifestations. Ivacaftor (IVA) and ivacaftor-lumacaftor (LUMA) combination are two new breakthrough CF drugs that directly modulate the activity and trafficking of the defective CFTR-protein. However, there is still a dearth of understanding on pharmacokinetic/pharmacodynamic parameters and the pharmacology of ivacaftor and lumacaftor. The HPLC-MS technique for the simultaneous analysis of the concentrations of ivacaftor, hydroxymethyl-ivacaftor, ivacaftor-carboxylate, and lumacaftor in biological fluids in patients receiving standard ivacaftor or ivacaftor-lumacaftor combination therapy has previously been developed by our group and partially validated to FDA standards. However, to allow the high-throughput analysis of a larger number of patient samples, our group has optimized the reported method through the use of a smaller pore size reverse-phase chromatography column (2.6 µm, C8 100 Å; 50 x 2.1 mm) and a gradient solvent system (0-1 min: 40% B; 1-2 min: 40-70% B; 2-2.7 min: held at 70% B; 2.7-2.8 min: 70-90% B; 2.8-4.0 min: 90% B washing; 4.0-4.1 min: 90-40% B; 4.1-6.0 min: held at 40% B) instead of an isocratic elution. The goal of this study was to reduce the HPLC-MS analysis time per sample dramatically from ~15 min to only 6 min per sample, which is essential for the analysis of a large amount of patient samples. This expedient method will be of considerable utility for studies into the exposure-response relationships of these breakthrough CF drugs.

Video Link

The video component of this article can be found at https://www.jove.com/video/56084/

Introduction

Cystic fibrosis (CF) is a common genetic disease involving the exocrine mucus glands of the lung, liver, pancreas, and intestines causing progressive multi-organ failure, such as a decline in lung function and pancreatic insufficiency[1,2,3]. Ivacaftor (IVA) is the first Food and Drug Administration (FDA-US) and European Medicines Agency (EMA) approved cystic fibrosis trans-membrane conductance regulator (CFTR) potentiator drug, with evidenced clinical efficacy producing a significant improvement in the lung function over placebo in a small subset of CF patients bearing the G551D-CFTR [glycine (G) in position 551 is replaced by aspartic acid (D)] missense mutation (~4-5% of the CF population)[4,5]. This orally administered drug increases the CFTR channel opening, thus increasing the chloride ion flow and acting on the primary defect that leads to the clinical manifestations of CF[4,6]. Unfortunately, IVA monotherapy is not effective in patients with the more common homozygous F508del mutation [in frame deletion of the CFTR gene which results in the loss of phenylalanine (F) at position 508] which results in misfolded CFTR, which is seen in ~50% of the CF population[7,8].

Recently, the FDA has granted approval for combining IVA with the CFTR corrector drug lumacaftor. The clever strategy of combining a CFTR corrector (lumacaftor, LUMA) which rescues F508del-CFTR to the cell surface with a modulator (IVA) which potentiates CFTR channel activity, effectively expands the treatment window to most of the CF population[5]. Questions remain over whether these drugs will fulfill their promise as a number of conflicting reports have emerged that cast doubt upon their clinical efficacy[9,10]. Additionally, improvements in lung function were only modest (2.6-4% for ivacaftor-lumacaftor combination) compared to the success achieved with IVA monotherapy in patients bearing a G551D mutation (10.6-12.5%)[8]. Potential antagonistic drug-drug interactions between IVA and LUMA that potentially limit the clinical efficacy of ivacaftor-lumacaftor combination come from its less than ideal pharmacokinetic properties[7,11]. IVA is extensively metabolized by cytochrome P450 enzymes (CYP), primarily to an active metabolite hydroxymethyl-IVA (IVA-M1, M1) and an inactive form IVA-carboxylate (IVA-M6, M6)[7,12]. The CYP3A4 inducer LUMA, on the other hand, is not extensively metabolized and is largely excreted unchanged in the feces[11]. As CYP3A4 inducers induce cytochrome metabolism, ivacaftor (CYP3A4 substrate) concentrations could be reduced. Moreover, both IVA and LUMA are very hydrophobic molecules and are ~99% bound to plasma proteins, which significantly limits the free (active) drug concentration[1,13].

Collectively, these factors may be coming together to limit the clinical efficacy of ivacaftor-lumacaftor combination. It is not known whether optimal plasma concentrations are achieved under the current dosage regimen for ivacaftor-lumacaftor combination or if the therapeutic threshold is maintained[8]. Presently, there is a paucity of information regarding pharmacokinetic parameters such as the peak and steady-state plasma concentrations of ivacaftor or ivacaftor-lumacaftor. Given the noted metabolism of ivacaftor and lumacaftor, monitoring of exposure-response relationships is requisite to achieve optimal dosage regimens for ivacaftor or ivacaftor-lumacaftor therapy. Our group recently published the first HPLC/LC-MS method for the monitoring of exposure-response relationships of IVA and LUMA[14]. No alternative techniques of measuring the concentrations of ivacaftor, its metabolites, and lumacaftor have been reported to date. To allow the high-throughput analysis of a larger patient collective and to dramatically reduce analysis time, our group has optimized the reported method through the use of a smaller pore size reverse-phase chromatography column and a gradient solvent system that reduces cost and running times.

Protocol

Approval for ethics was obtained from Monash University Human Research Ethics Committee (MUHREC).

1. Application of the Assay: Patient Sample Collection

1. Record the time when the patient takes their standard dose of either 150 mg ivacaftor or ivacaftor 125 mg /lumacaftor 200 mg.
2. Note down the exact time when the patient blood sample is collected.
   NOTE: We recommend collecting 4-5 samples over a 24 h time course. If the collection of only one sample is possible, the indicated time point is 2.5-4 h post ivacaftor or ivacaftor-lumacaftor combination dosing as C_{max} concentrations at steady state will be achievable after >5 days of consecutive treatment.
3. Collect 4-5 mL of blood in commercially available untreated blue tubes.
4. After collection of the whole blood, allow the blood to clot by leaving it undisturbed at room temperature.
   NOTE: This usually takes 15-30 min.
5. Remove the clot by centrifuging at 1,000-2,000 x g for 10 min at 4 °C in a refrigerated centrifuge. Designate the resulting supernatant as plasma.
6. Following centrifugation, immediately transfer the supernatant (plasma) into a clean polypropylene tube using a Pasteur pipette. Label the plasma tube with the Patient ID (*e.g.*, Patient 1), the administered drug (*e.g.*, ivacaftor-lumacaftor combination), and time the patient sample was collected (*e.g.*, 2.5 h post-dosage).
7. Maintain samples at 2-8 °C during handling. Once handling is completed, store samples at –20 °C.
8. If necessary, ship samples with an approved courier to the analyzing institute.
   NOTE: Samples are to be shipped on dry ice until arrival and should be kept at –80°C until analysis.

2. Preparation and Processing of Incurred Samples and Standards

NOTE: Plasma from healthy donors naïve to ivacaftor/lumacaftor therapy was obtained from the Australian Red Cross. To ensure the integrity, all samples/standards should be kept at 2-8 °C during collection and processing. Once handling is completed, store samples at –20 °C.

1. Weigh out IVA, IVA-carboxylate, hydroxymethyl-IVA, and LUMA which will serve as a reference for the internal standards.
2. Prepare two independent stock solutions of each analyte (IVA, M1, M6 and LUMA) in LC-MS grade methanol at 100 µg/mL and at 10 µg/mL.
   Note: Compounds will expire after 10 days on the benchtop.
3. Freshly prepare LC-MS calibration standards prior to each analytical run by dilution of the calibration standards stock solutions into human plasma to achieve following concentrations: 0.01, 0.025, 0.05, 0.1, 0.2, 0.5, 1.0, 2.5, 5.0, and 10.0 µg/mL. These samples are referred to as standards.
4. To precipitate proteins, prepare 0.1% formic acid (FA) in acetonitrile (ACN, LC-MS grade) and leave in the refrigerator until needed.

3. Pre-treatment of Incurred Samples and Standards

1. Allow both patient and blank plasma samples to equilibrate to room temperature.
2. Vortex to mix each plasma sample for 15 s per sample.
3. Transfer a 100 µL aliquot of either blank plasma for standards or patient samples for analysis into a 1.5-mL polypropylene microcentrifuge tube.
4. Add the internal standard IVA into each of the standard tubes (*e.g.*, 0.01, 0.025, 0.05, 0.1, 0.2, 0.5, 1.0, 2.5, 5.0, and 10.0 µg/mL to cover the potential concentration range), close the lid, and vortex for 2-3 s. Note that internal standard is only added to the blank plasma and no internal standard is added to the patient samples.
5. Repeat step 3.4. for the other internal standards M1, M6, and LUMA.
6. Spin down each tube briefly to ensure that there are no droplets on the lid.
7. Add 200 µL of the mix of 0.1% FA in ACN into each tube to precipitate plasma proteins.
8. Vortex the mixture vigorously for about 15 s.
9. Allow tubes to stand for 10 min in the refrigerator.
10. Centrifuge at 10,000 x *g* for 10 min at 4 °C, if possible.
11. Filter a 200 µL aliquot of the supernatant through a 13 mm syringe filter into a 1.5 mL HPLC vial.
12. Transfer 100 µL of the supernatant into the LC-MS vial for the HPLC-MS analysis.

4. HPLC-MS Analysis

NOTE: The HPLC-MS analysis was performed on an LC-MS system coupled with the triple quadrupole mass spectrometer (**Table 1**).

1. Turn on the LC-MS system, place the tray with the samples in the auto-sampler.
2. Attach the column to a guard column and connect to the LC-MS system.
3. Attach both bottles of mobile phases (bottle A: 100% ACN; bottle B: 0.1% formic acid in water) and equilibrate the LC-MS system.
4. **Incorporate the following parameters into the LC-MS protocol.**
    1. Split the mobile phase flow before entering the mass spectrometer in a 2:1 ratio (waste: MS inlet).
    2. Perform a gradient elution at a flow rate of 0.5 mL/min using a mobile phase consisting of 100% ACN and 0.1% formic acid in water (starting point at 40:60, v/v). Note that the volume percent of the mobile phase changes throughout analysis.
    3. Operate the mass spectrometer in a positive electrospray ionization mode.
    4. Make sure that the LC-MS settings are as follows: ion spray voltage 4.5 kV, collision energy 295.9 V, nebulizing gas: nitrogen at 3 L/min; collision gas: argon; drying gas flow 20 L/min, lens voltage Q3: - 22 V, desolvation temperature 250 °C with a heat block temperature of 400 °C.
    5. Inject 10 µL volume per sample.
    6. Detect analytes using multiple reaction monitoring (MRM). Monitor the ion transitions of *m/z* 392.49 → 393, *m/z* 408.49 → 409, *m/z* 422.47 → 423 and *m/z* 452.40 → 453 for IVA, IVA-M1, IVA-M6 and LUMA, respectively.
    NOTE: The newly optimized method has yet to be fully validated according to FDA standards.

5. Calibration Curves

1. Construct the LC-MS calibration curves before each analytical run using the relationship between the peak area ratios of each of the four analytes to internal standard and the calibration standard nominal concentrations of ivacaftor (IVA-M1, IVA-M6 or LUMA) within the LC-MS settings program.
NOTE: The exact steps for the construction of the calibration curve is dependent on the model of LC-MS equipment being used. This information may be available in the equipment manual.
2. Perform linear least-squares regression analysis by weighting $1/C^2$ according to the reciprocal of concentrations.

Representative Results

We have recently reported a method, partially validated to FDA standards, on a triple-quadrupole LC-MS and an HPLC detector system, using a C8 column (5 µm, 3.9 mm x 50 mm i.d.) with the mobile phase consisting of 100% ACN and 0.1% formic acid in water (40:60, v/v) at a flow rate of 1 mL/min. A linear correlation of the peaks was observed over a concentration range from 0.01 to 10 µg/mL in human plasma for all metabolites, ivacaftor, Iva-M1, Iva-M6, and lumacaftor[14]. Here this method has been optimized to allow dramatically reduced LC retention times and, therefore, the complete running time of the assay, which is essential for the high-throughput analysis of a large amount of clinical samples. The assay was optimized through the use of a smaller pore size reverse-phase chromatography column and a gradient solvent system instead of an isocratic elution, reducing the time per sample from ~15 min to only 6 min. The analytes are determined in electrospray positive mode using multiple reaction monitoring (MRM). The procedure should be validated in-house prior to use, as we have previously detailed[14].

The accuracy of the method was 94.2% ± 4.53% to 97.5% ± 2.94%, depending on the analyte[14]. The calibration range from 0.01 to 10 µg/mL is applicable for use in the clinical setting, in particular given that under ivacaftor-lumacaftor therapy, very low concentrations of active IVA and M1 were reported by our group previously, in plasma potentially due to the induction of CYP3A4 by LUMA, which results in extensive IVA metabolism[14]. The intra-day accuracy of each analyte was assessed with six independently prepared quality control (QC) samples on the same day at concentrations of 0.05, 0.5, and 8 µg/mL. The inter-day accuracy was assessed with six independently prepared QC samples on three consecutive days. Accuracy and precision were calculated via relative standard deviation (RSD). For each QC sample, the RSD values should be less than 15%[15]. All four analytes showed RSD values of less than 15%[14]. The lower limit of detection (LOD) and quantification (LOQ) were established: for IVA LOD 2.50 x 10^{-3} µg/mL and LOQ 7.57 x 10^{-3} µg/mL; for M1, LOD 4.57 x 10^{-4} µg/mL and LOQ 1.38 x 10^{-3} µg/mL; for M6 LOD 5.86 x 10^{-4} µg/mL and LOQ 1.78 x 10^{-3} µg/mL; for LUMA LOD was 6.08 x 10^{-4} µg/mL and LOQ 1.84 x 10^{-3} µg/mL[14]. A typical chromatogram is shown in **Figure 1**. An optimized chromatographic resolution was obtained using a mobile phase of 100% ACN and 0.1% formic acid in water (starting point at 40:60, v/v) with a gradient elution on a C8 column (2.6 µm; 100 Å; 50 mm x 2.1 mm). The gradient separation was used with 40% of mobile phase B from 0 to 1 min; then 40%-70% of mobile phase B from 1 min to 2 min; holding 70% of mobile phase B from 2 min to 2.7 min; increasing from 70% to 90% of mobile phase B from 2.7 min to 2.8 min; holding 90% of mobile phase B from 2.8 min to 4.0 min for washing purposes; finally returning from 90% to 40% of mobile B from 4.0 min to 4.1 min; then holding 40% of mobile phase B from 4.1 min to 6.0 min for initial conditions (**Figure 1**). The LC retention times were as follows for M6 1.0 min; for M1 1.3 min; for IVA: 1.55 min; for LUMA: 2.3 min (**Figure 1**). 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 lumacaftor.

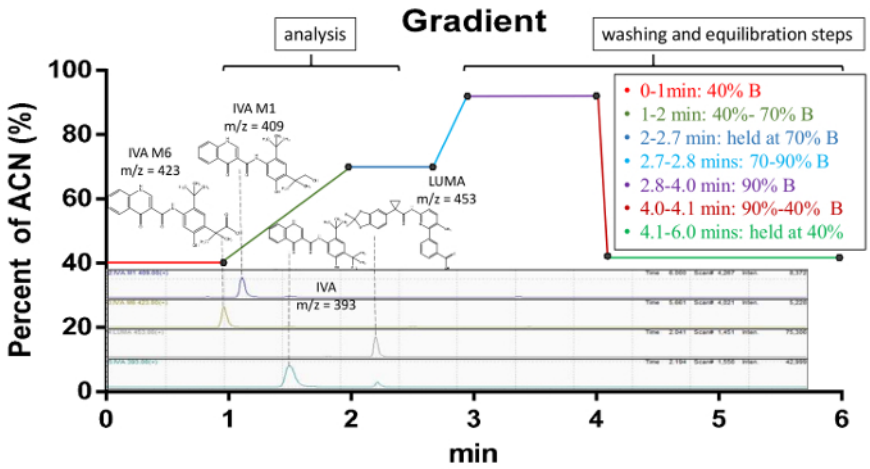

Figure 1: Representative LC-MS chromatogram of human plasma spiked with 10 µg/mL of IVA-M6, IVA-M1, lumacaftor, and ivacaftor in relation to the gradient elution of pump. A 0.1% formic acid in water and pump B 100% acetonitrile (0-1 min: 40% B; 1-2 min: 40%-70% B; 2-2.7 min: held at 70% B; 2.7-2.8 min: 70%-90% B; 3.8-4.0 min: 90% B washing; 4.0-4.1 min: 90%-40% B; 4.1-6.0 min: held at 40% B). The ion transitions of *m/z* 392.49 → 393, *m/z* 408.49 → 409, *m/z* 422.47 → 423 and *m/z* 452.40 → 453 were used for MS/MS monitoring of ivacaftor, IVA- M1, IVA-M6 and lumacaftor, respectively. Please click here to view a larger version of this figure.

HPLC Conditions	
Column	C8; 2.6 µm; 100 Å; 50 x 2.1 mm
Guard column	HPLC In-Line Filter 0.5 µm; Depth Filter x 0.004 inID
	Filter x 0.004 inID
Column temperature:	30 °C
Mobile phase A:	0.1 % FA in water
Mobile phase B:	100 % ACN
Mobile Phase composition at starting point	60 / 40 (v/v)
Sample temperature:	4 °C
Injection volume:	5 µL
Needle wash	80 % methanol, 20 % water 300 µL
Flow rate:	0.5 mL/min
Gradient:	- 1 min: 40 % B
	- 1 - 2 min: 40 % - 70 % B
	- 2 - 2.7 min: held at 70 % B
	- 2.7 - 2.8 min: 70 % - 90 % B
	- 2.8 - 4.0 min: 90 % B washing
	- 4.0 - 4.1 min: 90 % - 40 %
	- 4.1 - 6.0 min: held at 40 %,
Total run:	6 mins
Retention times:	Retention time for M6: 1.0 min
	Retention time for M1: 1.3 min
	Retention time for IVA: 1.55 min
	Retention time for LUMA: 2.3 min
MS Conditions	
Detection mode:	electrospray positive ESI +
Ion spray voltage:	4.5 kV
Collision Energy:	295.9 V
Nebulizing Gas (nitrogen):	3 L/min
CID Gas:	230 kPa
Drying gas (nitrogen) flow:	20 L/min
Q1 Pre-rod bias:	- 5 V
CE:	- 25 V
Q3 pre-rod bias:	- 5 V
Interface current:	0.1 UA
Desolvation temperature:	250 °C
Heat block temperature:	400 °C
IVA m/z	392.49 → 393
M1 m/z	408.49 → 409
M6 m/z	422.47 → 423
LUMA m/z	452.40 → 453

Table 1: Details of the HPLC-MS conditions.

Discussion

As previously reported, our group has for the first time developed and validated a HPLC and LC-MS method for rapid detection and quantification of ivacaftor and its major metabolites hydroxymethyl-IVA M1 (active) and IVA-carboxylate M6 (inactive); and lumacaftor in the plasma and sputum of CF patients[14]. The assay reported by our group previously was successfully used to quantify the concentration of LUMA, IVA, IVA-M1, and IVA-M6 in the plasma and sputum of CF patients undergoing steady state standard therapy with IVA 150 mg /q12 h (Latin terminology: *quaque* 12 h; once every 12 h) or 200 mg /q12 h LUMA-125 mg /q12 h IVA combination[14]. For the high-throughput analysis of large amounts of patient samples, we have optimized the reported method by using a smaller pore size reverse-phase chromatography column C8 (2.6 µm; 100 Å; 50 x 2.1 mm^2) and a gradient solvent system instead of the current isocratic elution. This reduces the running time to 6 min per sample.

This reliable and novel method offers a simple, sensitive and rapid approach for therapeutic drug monitoring of ivacaftor and ivacaftor-lumacaftor combination in biological fluids. Given the need to develop exposure-response relationships to maximize drug efficacy as well as contain health care costs, more sensitive tools need to be developed and validated to assist clinicians in using evidence-based therapies and high-throughput analytical techniques for patients suffering from CF. Our group has previously reported a partially validated HPLC-MS for simultaneously analyzing IVA, IVA-M1, IVA-M6, and LUMA in the plasma and sputum of CF patients[14]. Establishing a common and easy-to-use analytical protocol for the analysis of patients receiving ivacaftor or ivacaftor-lumacaftor combination would facilitate data comparison and interpretation of results of future pharmacokinetic studies. Measuring IVA, IVA-M1, IVA-M6, and LUMA in biological fluids using LC-MS techniques might embody a powerful tool for exploring the exposure-response relationship in relation to therapeutic outcomes.

More generally, HPLC-MS analysis of ivacaftor or ivacaftor-lumacaftor combination in biological fluids might provide a molecular basis for the implementation of individualized pharmacotherapeutic strategies with patients suffering from CF and may prove useful on the grounds of making the expensive ivacaftor or ivacaftor-lumacaftor combination therapy more cost effective, by potentially indicating the need for less frequent dosing. By applying the proposed analytical method to clinical pharmacokinetic/pharmacodynamic studies, the opportunity has arisen to further investigate the pharmacokinetic parameters of ivacaftor or ivacaftor-lumacaftor combination on a larger patient collective.

Disclosures

The authors have nothing to disclose.

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