M0930-06-38

QUANTITATION OF BEMNIFOSBUVIR AND METABOLITES IN HUMAN BRONCHOALVEOLAR LAVAGE (BAL) BY LC-MS/MS

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PURPOSE

Bemnifosbuvir (BEM, AT-527) is a novel oral nucleotide antiviral drug candidate currently in Phase 3 clinical development for the treatment of COVID-19.

Bronchoalveolar lavage (BAL) is a minimally invasive sampling procedure for the evaluation of lower respiratory tract pathology and direct measurement of drug levels in the pulmonary epithelial lining fluid (ELF). The latter is critical to the treatment optimization of respiratory diseases such as COVID-19 using direct-acting antivirals.

This poster outlines the challenges and strategies related to the analysis of compounds in BAL.

OBJECTIVE(S)

The primary objective of this project was to determine the concentrations of AT-511, the free base of AT-527, and its metabolites AT-551, AT-229, and AT-273 in ELF after administration of multiple doses of BEM in healthy subjects. BAL collection consists of administering measured volumes of warmed saline solution into targeted pulmonary regions to collect ELF via aspiration of the lavage solution. The resulting BAL sample contained diluted (ca. 100-fold) ELF as an aqueous medium. Non-specific binding (NSB) within storage containers, physicochemical disparities among the four analytes in terms of solubility, stability, and specificity as well as low targeted LOQs (limit of quantitation) due to dilution of the ELF were all key factors in the development of a sensitive and rugged assay combining sample collection, extraction, chromatographic resolution, and mass spectrometric detection.

METHOD(S)

BAL samples were collected and mixed with an anti-adsorptive agent solution before storage. Following deproteinization with cold acetonitrile: methanol (1:3), samples were subsequently diluted for injection. Chromatographic separation involved gradient elution on an XBridge C18 column (50 x 4.6mm, 3.5 µm) using 10 mM ammonium bicarbonate and acetonitrile: methanol (1:1) as an organic modifier. The LC system was coupled to a SCIEX API 5000 mass spectrometer operated in positive electrospray ionization mode monitoring the MRM transitions 582.2>376.2 (AT-511), 313.1>148.2 (AT-229), 464.1>375.2 (AT-551) and 300.1>152.2 (AT-273). Analytical ranges were 0.200 - 200 ng/mL for AT-511 and AT-229 and 0.400 - 400 ng/mL for AT-551 and AT-273.

RESULT(S)

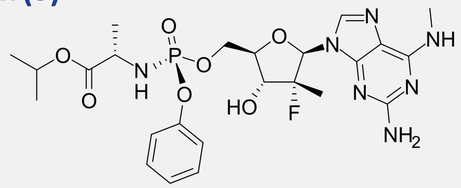

Figure 1. Structure of bemnifosbuvir

Non-Specific Binding

BAL contains diluted (ca. 100-fold) epithelial lining fluid (ELF) as an aqueous medium. Lipophilic compounds are prone to non-specific binding (NSB) within storage containers. NSB evaluation is tabulated in ***Table 1*** and reveals significant recovery loss for AT-511 from non-treated BAL.

Table 1. NSB evaluation in non-treated BAL

Concentration	% Recovery from Untreated BAL			
	AT-511	AT-551	AT-273	AT-229
1.0 ng/mL	70.6	115.3	93.3	101.7
1.0 µg/mL	60.2	112.6	98.2	92.3

Sample treatment with an anti-adsorptive agent (reagent alcohol 1:1 v:v) improves recovery from storage containers and allows for quantitative analysis of all 4 analytes, as displayed in Table 2

Table 2. NSB evaluation in treated BAL

Concentration	% Recovery from BAL Treated with Reagent Alcohol			
	AT-511	AT-551	AT-273	AT-229
1.0 ng/mL	102.5	98.3	89.3	102.0
1.0 µg/mL	96.9	97.9	100.1	99.9

Extraction and LC-MS Optimization

As the BAL protocol resulted in significant dilution of the ELF, overall extract dilution was minimized to achieve the target LOQs ***(Figure 2)***. Due to polarity differences between analytes, extraction involved non-discriminatory protein precipitation.

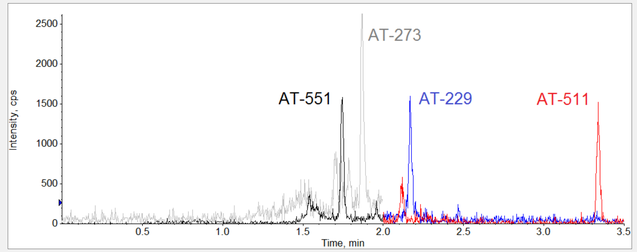

Figure 2. Extracted LOQ from BAL

Chromatographic conditions were developed in reversed-phase mode on an XBridge C18 analytical column. The use of ammonium bicarbonate as a modifier greatly improved the signal-to-noise ratio compared to traditional acidic modifiers (i.e. formic acid, acetic acid). Gradient elution with a mixture of acetonitrile and methanol (1:1) yielded excellent peak shape and baseline separation of all four analytes within 3.5 minutes. Significant carry-over was observed and mitigated with the implementation of a needle seat back-flush procedure using a second binary HPLC pump.

MS acquisition was divided into two periods to optimize the dwell times of all eight compounds (4 analytes and their respective deuterated internal standards), thereby improving the signal-to-noise ratio through optimization of the duty cycle for each analyte.

Method Qualification

Calibration curves were constructed with ranges of 0.200-200 ng/mL (AT-511 and AT-229) and 0.400-400 ng/mL (AT-551 and AT-273), using peak area ratio to their respective IS and applying a weighed (1/x2) linear regression. The precision and accuracy of the method were evaluated after replicate analysis of four quality control (QC) levels (***Table 3***).

Recovery of each analyte from BAL ranged from 92.8% to 104.2%. Specificity was evaluated from 7 different human BAL donors which were found to be free of significant interference. The matrix effect (***Table 4***) and matrix factor were evaluated on 6 different donors at low and high QC concentrations. Short-term stability (18h) and long-term stability (8 days) were also assessed.

Table 3. Intra-run precision and accuracy (6 replicates)

Analyte	Parameter	QC LOQ	Low QC	Mid QC	High QC
AT-511	%CV	4.9	6.5	1.3	1.7
	% Nominal	102.6	97.7	97.6	98.8
AT-229	%CV	5.0	4.0	1.4	2.0
	% Nominal	102.0	99.8	99.3	97.5
AT-551	%CV	9.4	4.6	3.2	2.5
	% Nominal	99.6	97.8	101.0	99.6
AT-273	%CV	8.8	3.3	3.9	3.4
	% Nominal	98.6	90.6	100.9	97.1

Table 4. Matrix effect evaluation (6 donors – 3 replicates)

Analyte	Parameter	Low QC	High QC
AT-511	%CV	9.7	3.8
	% Nominal	107.0	97.6
AT-229	%CV	6.8	5.2
	% Nominal	106.3	109.4
AT-551	%CV	5.0	3.9
	% Nominal	97.3	102.5
AT-273	%CV	3.9	3.9
	% Nominal	101.5	92.0

Epithelial Lining Fluid (ELF) Recovery

Despite the enormous utility of BAL as a technique to evaluate molecular components of the ELF, the recovered solutions are a variable mixture of saline, ELF, and ELF components.

To estimate the recovered ELF in the BAL mixture, urea was analyzed in both BAL and plasma, at the corresponding sampling times, and used as a marker of dilution. The measured urea concentration ratio between BAL and plasma was then used as a correction factor to calculate AT-511 and its metabolite concentrations in ELF.

CONCLUSION(S)

A bioanalytical method for the determination of AT-511 (free base of BEM/AT-527) and its metabolites AT-551, AT-229, and AT-273 in bronchoalveolar lavage (BAL) was successfully developed and qualified.

The method is linear, precise, and accurate, and was applied for the determination of all four analytes in epithelial lining fluid (ELF) after administration of multiple doses of Bemnifosbuvir.

Antiviral drug levels of BEM were consistently achieved in the lungs with BEM 550 mg BID. Mean ELF levels of AT-273, a surrogate of the active triphosphate metabolite of the drug, were 0.62 and 0.46 µM (185 and 138 ng/mL) respectively at 4 and 12h, exceeding/approaching the target 0.5µM, EC90 of the drug inhibiting SARS-CoV-2 replication in human airway epithelial cells.

ALTASCIENCES

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