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Published in:
Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences

DOI:
10.1016/j.jchromb.2024.124244

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2024

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Abd-Elaziz, K. S., Cheng, R., Chen, J., Maarse, H., Lee, Y., Yang, W., Chien, B., Diamant, Z., Kosterink, J., & Touw, D. J. (2024). Validation of a method for the determination of Aderamastat (FP-025) in K 2EDTA human plasma by LC-MS/MS. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *1245*, Article 124244. https://doi.org/10.1016/j.jchromb.2024.124244

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Download date: 17-03-2025

Journal of Chromatography B 1245 (2024) 124244

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

Validation of a method for the determination of Aderamastat (FP-025) in K_2EDTA human plasma by LC-MS/MS

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ARTICLE INFO

Keywords:
Aderamastat
FP-025
Matrix Metalloproteinase (MMP)-12 inhibitor
LC-MS/MS
Bioanalysis Assay
Validation

ABSTRACT

Aderamastat (FP-025) is a small molecule, selective matrix metalloproteinase (MMP)-12 inhibitor, under development for respiratory conditions which may include chronic inflammatory airway diseases and pulmonary fibrosis.

To support evaluation of the pharmacokinetic parameters of Aderamastat in humans, we developed and validated a high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS) analytical method for the quantification of Aderamastat in human plasma. This assay was validated in compliance with the Food and Drug Administration (FDA) Good Laboratory Practice Regulations (GLP) and European Medicines Agency (EMA) guidelines.

K_2EDTA human plasma samples were spiked with internal standard, processed by liquid–liquid extraction, and analyzed using reversed-phase HPLC with Turbo Ion Spray® MS/MS detection. Separation was done using a chromatographic gradient on 5 μm C6-Phenyl 110 Å, 50*2 mm analytical column at a temperature of 35 °C.

The LC-MS/MS bioanalytical method, developed by QPS Taiwan to determine the concentration of Aderamastat in K_2EDTA human plasma, was successfully validated with respect to linearity, sensitivity, accuracy, precision, dilution, selectivity, hemolyzed plasma, lipemic plasma, batch size, recovery, matrix effect, and carry-over. These data indicate that the method for determination of Aderamastat concentrations in human K_2EDTA plasma can be used in pharmacokinetics studies and subsequent clinical trials with Aderamastat.

Authors declare that, this novel data is not published and not under consideration for publication by another journal than this journal. All data will be made available on request.

1. Background

Matrix metalloproteinases (MMPs) are proteases with a vast variety of biological and pathophysiological activities, and several of them have been linked to various diseases [1–5]. Although perceived as potential therapeutic targets, several development programs of MMP inhibitors have been discontinued due to serious adverse events caused by non-selective inhibition of MMPs [6–8], as they share similar substrates [9].

Among the currently over 20 known zinc-protease MMPs, especially MMP-2, MMP-8, MMP-9 and MMP-12, have been shown to be involved in the pathophysiology of pulmonary disorders including asthma, chronic obstructive pulmonary disease (COPD) and pulmonary fibrosis [10]. MMP-12 is a 54-kDa proenzyme, which is processed into a 45-kDa and end in a 22-kDa active protease [11]. MMP-12 is predominantly identified in alveolar macrophages, hence known as macrophage elastase [12], but also excreted by bronchial epithelial cells [13] and airway

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https://doi.org/10.1016/j.jchromb.2024.124244
Received 17 March 2024; Received in revised form 6 June 2024; Accepted 15 July 2024
Available online 18 July 2024
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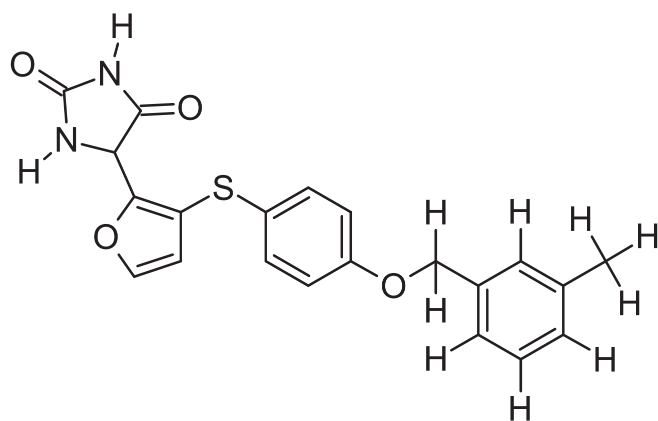

Fig. 1a. Chemical structure of FP–025.

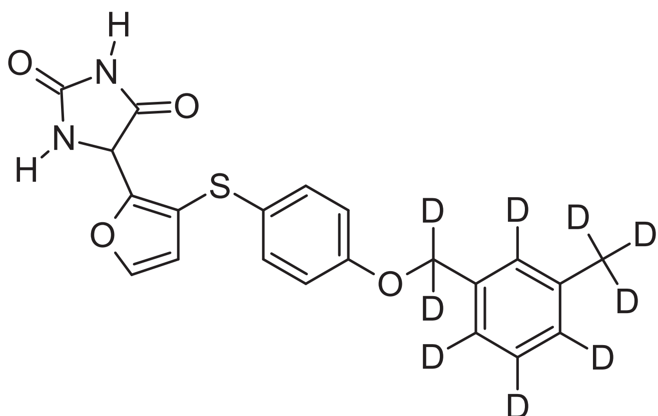

Fig. 1b. Chemical structure of internal standard (IS).

Table 1
Gradient program (time and compositions of mobile phase a and mobile phase b).

Program	Gradient						
Time (min)	1.0	1.5	2.0	2.2	2.8	3.0	3.5
%mobile phase B	60	75	75	95	95	60	Stop
Flow (mL/min)	0.3	0.3	0.3	0.3	0.3	0.3	

Table 2a
Regression Analysis of Aderamastat (FP-025) in K_2EDTA Human Plasma.

Run ID[a]	Slope	Intercept	R-Squared
1	0.002592571	0.001965165	0.9926
3	0.002726672	0.000735520	0.9894
4	0.002749581	0.001762039	0.9948
8	0.002741810	0.001531864	0.9906
9	0.002588123	0.000960983	0.9935
11	0.001146859	−0.000007894	0.9966

[a] Linear regression analysis with $1/x^2$ weighting. ID=identification.

smooth muscle cells [14]. Accumulated evidence derived from animal models and human data shows that MMP-12 is involved in type 2 inflammation acting on proliferation and migration of inflammatory cells [15] as well as in tissue remodeling through its ability to turn over extracellular matrix components, mainly elastin [16].

Aderamastat (FP-025) is a novel selective non-hydroxamate, small molecule inhibitor of MMP-12 (2), with a 90-fold selectivity over the next closest family member (MMP-2) and with two to three orders of magnitude selectivity over seven other MMP family members, i.e.: MMP-1, MMP-3, MMP-7, MMP-8, MMP-9, MMP-13, and MMP-14 (2).

Presently, Aderamastat is being developed as a medicinal product for the treatment of asthma, COPD and pulmonary fibrosis. In several clinical trials, Aderamastat was found to be safe and tolerable in doses up to a daily dose of 800 mg (2) and showed a favorable pharmacokinetics profile in both healthy volunteers and in asthma patients (2).

Determination and measurement of human plasma concentration of investigational medicinal products is crucial for clinical trials executions and for clinical applicability in later stages.

The aim of this study was to develop and validate a method to determine Aderamastat in K_2EDTA human plasma using LC-MS/MS in order to measure drug exposure in humans. For our best knowledge, no early LC-MS/MS bioanalytical determination method for Aderamastat has been developed and the current assay will be demonstrated and discussed for the first time.

2. Materials and methods

2.1. Materials

The analytical method was developed and validated at QPS Taiwan to quantify Aderamastat in K_2EDTA human plasma. Aderamastat is C21H18N2O4S with a molecular weight of 394.44 g/mole (Fig. 1a). The stable isotope labeled internal standard (IS) was FP–025–d9 (C21H9D9N2O4S) with a molecular weight of 403.50 g/mole (Fig. 1b). The concentration range was designed to determine the exposure that yields a safe, tolerable and effective dose for the later phases of clinical trials.

Aderamastat was provided by Foresee Pharmaceuticals Co., Ltd (Taipei, Taiwan). High-performance liquid chromatography (HPLC) grade quality Methanol and Methyl *tert*-Butyl Ether (MtBE) were purchased from MACRON fine chemicals (Avantor Performance Materials, Inc., Center Valley, Pennsylvania, United States). HPLC grade quality acetonitrile was purchased from Spectrum Chemical Mfg. Corp (New Brunswick, New Jersey, United States). Formic acid was purchased from J.T. Baker (Phillipsburg, New Jersey, United States). Ammonium acetate was purchased from Sigma–Aldrich (St. Louis, Missouri, United States). 0.2 M ammonium acetate was used as buffer and was prepared by QPS Taiwan by dissolving 3.86 g of ammonium acetate in 250 mL Milli-Q water. Pooled K_2EDTA human plasma was used as matrix and was purchased from Bioreclamation IVT (West Sussex, United Kingdom). For stability experiments in whole blood, blood was freshly collected from healthy donors at QPS Taiwan (Taipei, Taiwan).

2.2. Preparation of stocks, standards, and quality controls

Two primary stocks (stock A) were prepared by two different QPS staff members. Using a 5 mL volumetric flask, 10.116 mg of Aderamastat was dissolved and the flask was filled with methanol. The second primary stock was prepared using 10.129 mg. The concentration of the Aderamastat solution was calculated using a purity of 0.989 and a correction factor of 1.000 for the Aderamastat form. A secondary stock (stock B) was prepared by dilution with methanol directly from the primary stock. The internal standard solution was prepared by dissolving 2.526 mg of FP-025-d9 in 5 ml volumetric flask and the flask was filled to volume with methanol. The concentration of the FP-025-d9 was calculated using a purity factor of 0.990 and a correction factor of 1.000 for the FP-025-d9 form. All solutions were stored at approximately −20 °C with precautions taken to prevent evaporative losses from the stock solutions during the study. Calibration curves and quality control (QC) samples were freshly and separately prepared in K_2EDTA human plasma. The calibration curves had the concentrations of 5.00, 10.0, 25.0, 50.0, 250, 500, 2500 and 5000 ng/mL, while the QC had concentrations of 5.00, 15.0, 1500 and 4000 ng/mL.

Table 2b
Back-Calculated Concentrations (ng/mL) of Aderamastat (FP-025) Calibration Standards in K_2EDTA Human Plasma.

Run ID	5.00	10.0	25.0	50.0	250	500	2500	5000
1	4.63	10.2	25.1	56.3	268	487	2320	4420
	5.01	10.8	25.4	*66.3	277	502	2350	4570
3	4.42	10.6	25.4	52.8	264	472	2280	4270
	5.05	10.8	26.4	57.0	278	500	2300	4480
4	4.85	10.2	24.5	53.6	272	493	2360	*4140
	4.95	10.2	25.5	56.1	239	495	2390	4420
8	4.66	10.7	25.8	55.3	267	484	2320	4320
	4.75	11.2	25.2	53.1	277	495	2270	4480
9	4.73	9.99	24.8	54.2	267	480	2350	4390
	4.94	10.9	25.5	54.6	272	505	2360	4520
11	4.82	10.7	24.5	50.9	265	497	2400	4800
	5.11	9.34	25.2	54.6	260	491	2500	4570
Mean	4.83	10.5	25.3	54.4	267	492	2350	4480
S.D.	0.200	0.503	0.534	1.77	10.5	9.58	62.1	143
%CV	4.1	4.8	2.1	3.3	3.9	1.9	2.6	3.2
%RE	−3.4	5.0	1.2	8.8	6.8	−1.6	−6.0	−10.4
n	12	12	12	11	12	12	12	11

SD=standard deviation, %CV=percent coefficient of variation, %RE=Percent Relative Error, n = number.
* rejected.

Table 3
Intraday and Interday Precision and Accuracy of Aderamastat (FP-025) in K_2EDTA Human Plasma.

Run ID	5.00(ng/mL)	15.0 (ng/mL)	1500(ng/mL)	4000(ng/mL)
1	4.79	13.2	1370	3950
	4.59	14.0	1400	4050
	4.69	14.3	1400	4140
	4.36	12.8	1400	4090
	4.10	13.3	1390	4060
	4.51	13.7	1390	4140
Intra-run Mean	4.51	13.6	1390	4070
S.D.	0.248	0.554	11.7	70.8
%CV	5.5	4.1	0.8	1.7
%RE	−9.8	−9.3	−7.3	1.8
n	6	6	6	6
3	4.60	13.6	1280	3770
	4.50	13.6	1330	3820
	4.71	13.5	1300	3760
	4.19	13.7	1330	3910
	4.27	13.1	1350	3830
	4.57	13.8	1320	3880
Intra-run Mean	4.47	13.6	1320	3830
S.D.	0.202	0.243	24.8	59.1
%CV	4.5	1.8	1.9	1.5
%RE	−10.6	−9.3	−12.0	−4.3
n	6	6	6	6
4	4.39	12.9	1290	3630
	4.36	13.0	1290	3710
	4.42	13.0	1290	3710
	4.06	#12.1	1290	3660
	4.07	#12.6	1300	3720
	4.00	13.8	1300	3690
Intra-run Mean	4.22	12.9	1290	3690
S.D.	0.192	0.559	5.16	35.0
%CV	4.5	4.3	0.4	0.9
%RE	−15.6	−14.0	−14.0	−7.8
n Inter-run	6	6	6	6
Mean	4.4	13.3	1330	3860
Inter-run S.D.	0.242	0.547	45.5	172
Inter-run %CV	5.5	4.1	3.4	4.5
Inter-run %RE	−12.0	−11.3	−11.3	−3.5
n	18	18	18	18

> 15|%RE| from Nominal. SD=standard deviation, %CV=percent coefficient of variation, %RE=Percent Relative Error, n = number.

2.3. Sample preparation

The standard solutions, QCs, blank matrix, and System Suitability Test (SST) Sample were thawed on wet ice and vortexed for approximately 1 min before pipetting. 95 µL of pooled human plasma was spiked into pre-labeled tubes for blank, blank + IS, carryover blank, SST sample, and calibration standards. Subsequently, 5 µL of 400 ng/mL spiking solution (Aderamastat) was added to the SST tube, 5 µL of standard spiking solutions at each concentration were added to calibration standards tubes and 5 µL of methanol was added to blank, carryover blank, and blank + IS labeled tubes. The mixtures were vortexed for approximately 1 min at high speed. From each QC samples concentration, 100 µL was added to the QC tubes.

To the blank matrix, SST samples, calibration standards and QC samples 50 µL of 1000 ng/mL IS (FP-025-d9 in methanol) was added, and 50 µL of methanol was added to blank and carryover blank respectively. All tubes were vortexed for approximately 1 min at high speed.

2.4. Sample extraction

To the prepared samples 200 µL of 0.2 M ammonium acetate was added and vortex mixed at high speed for approximately 1 min. After addition of 3 mL of MtBE, the tubes were vortex mixed at high speed for approximately 5 min. The tubes were centrifuged at 4 °C for 10 min at 3000 rpm. The tubes were placed in a dry ice/acetone mixture to freeze the aqueous layer. The supernatant was transferred into corresponding labeled new tubes and evaporated to dryness in a 40 °C water bath under a nitrogen stream for approximately 15 min. The samples were reconstituted with 500 µL of methanol:water(45:55, v:v) and vortex mixed at high speed for approximately 1 min. The solutions were transferred into a 96 well plate. The plate was covered with a seal and centrifuged at 4 °C for 5 min at 3000 rpm. 10 µl was automatically injected onto the LC-MS/MS system.

2.5. Equipment and chromatography conditions

Plasma samples were spiked with internal standard, processed by liquid–liquid extraction, and analyzed using reversed-phase HPLC with Turbo Ion Spray® MS/MS detection. Negative $(M-H)^-$ ions for Aderamastat and FP-025-d9 were monitored in MRM mode. Analyte-to-IS peak area ratios for the standards were used to create a linear calibration curve using $1/x^2$ weighted least-squares regression analysis.

LC-MS/MS analysis was performed using a Triple Quadrupole MS API 4000 (Applied Biosystems), a solvent delivery system LC-10AD, SCL-10Avp (Shimadzu Corp) and an auto injector HTC PAL (CTC Analytics).

Chromatography was performed on a Gemini® 5 µm C6-Phenyl 110 Å, 50*2 mm analytical column (Phenomenex) with 0.50——2.10 min to mass spectrum switching. The autosampler and the column oven

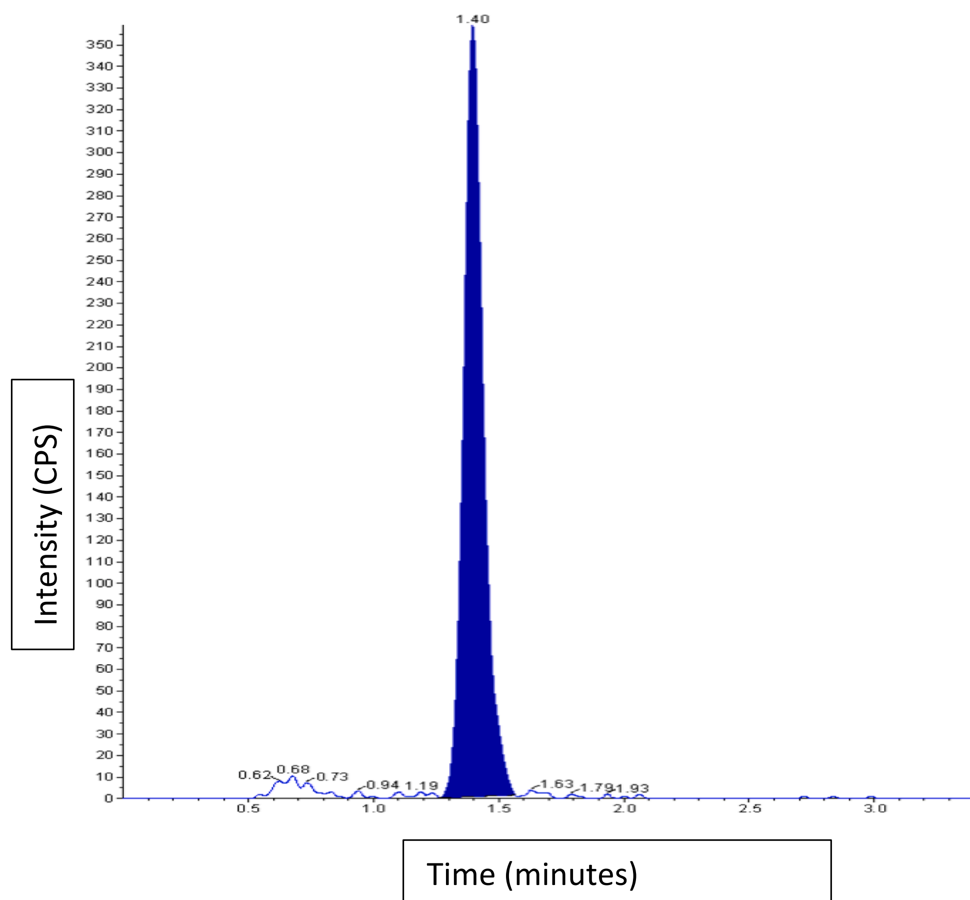

Fig. 2a. Mass Chromatograms of the lower limit of quantification (5 ng/mL) of Aderamastat in K_2EDTA Human Plasma.

temperatures were set at 4 °C and 35 °C, respectively. Elution gradient was performed using mobile phase A consisting of water:1M ammonium acetate (100:0.1, v:v) and mobile phase B consisting of acetonitrile:1M ammonium acetate (100:0.1, v:v). Flow rate was 300 µL/min. The gradient program (time and compositions of mobile phase A and mobile phase B) are provided in Table 1.

The autosampler injection volume was 10 µl. After each injection, the sampler manager was washed with water:formic acid (100:2, v:v) and acetonitrile:formic acid (100:0.1, v:v) before drawing a subsequent sample.

There was a minor response from the IS in the multiple reaction monitoring (MRM) trace of the analyte, at 17.4 % of the LLOQ response. There was also a minor response from the analyte in the MRM trace of the IS, at 0.1 % of the IS working concentration response.

2.6. Method validation

The method was validated according to FDA's 'Guidance for Industry Bioanalytical Method Validation' and EMA's 'Guideline on bioanalytical method validation', May 2001 [17,18]. Linearity, accuracy and precision, dilution linearity, selectivity and sensitivity, extraction recovery, matrix effect, hemolyzed plasma, lipemic plasma, batch size, injection carry over, stability of Aderamastat in K_2EDTA human plasma at 4 °C, through freeze/thaw cycles and following long-term storage (at −20 °C and −70 °C). Aderamastat stability was assessed in solvent (ambient temperature and −20 °C), in human whole blood and in processed samples.

2.6.1. Linearity, accuracy, and precision

The 8-point, at eight concentration levels (5, 10, 25, 50, 250, 500, 2500, and 5000 ng/mL) calibration curve was generated over six separate runs, where linearity was assessed on response. Precision and accuracy of the method were evaluated by analyzing QC samples at four different concentrations (5, 15, 1500, and 4000 ng/mL) within the standard curve range to validate reproducibility. The LLOQ QC was the same concentration as the lowest calibration standard, the next lowest QC concentration was ≤ 3 times the concentration of the lowest calibration standard, and the high QC was 75 % to 90 % of the highest calibration standard.

Precision was determined by the coefficient of variation (%CV). Precision was calculated according to the following equation:

$$Precision = \%CoefficientofVariation(\%CV) = \frac{StandardDeviation}{100} \times 100$$

Accuracy was determined by replicate analysis of samples containing known amounts of the analyte and expressed as percent relative error (% RE). Accuracy was calculated according to the following formula:

$$Accuracy = PercentRelativeError(\%RE) = \frac{DeterminedConcentration - NominalConcentration}{NominalConcentration} \times 100$$

2.6.2. Selectivity, sensitivity, and carry-over

The selectivity of this method was evaluated by extracting and analyzing six individual lots of blank K_2EDTA human plasma without either Aderamastat or the IS. The K_2EDTA whole blood collected at QPS Taiwan was frozen at −20 °C overnight, sonicated for approximately 20 min, and centrifuged at 4 °C for 10 min to harvest the grossly hemolyzed plasma. The grossly K_2EDTA hemolyzed plasma was then mixed with blank K_2EDTA human plasma obtained from Bioreclamation IVT to make the 2 % hemolyzed K_2EDTA human plasma.

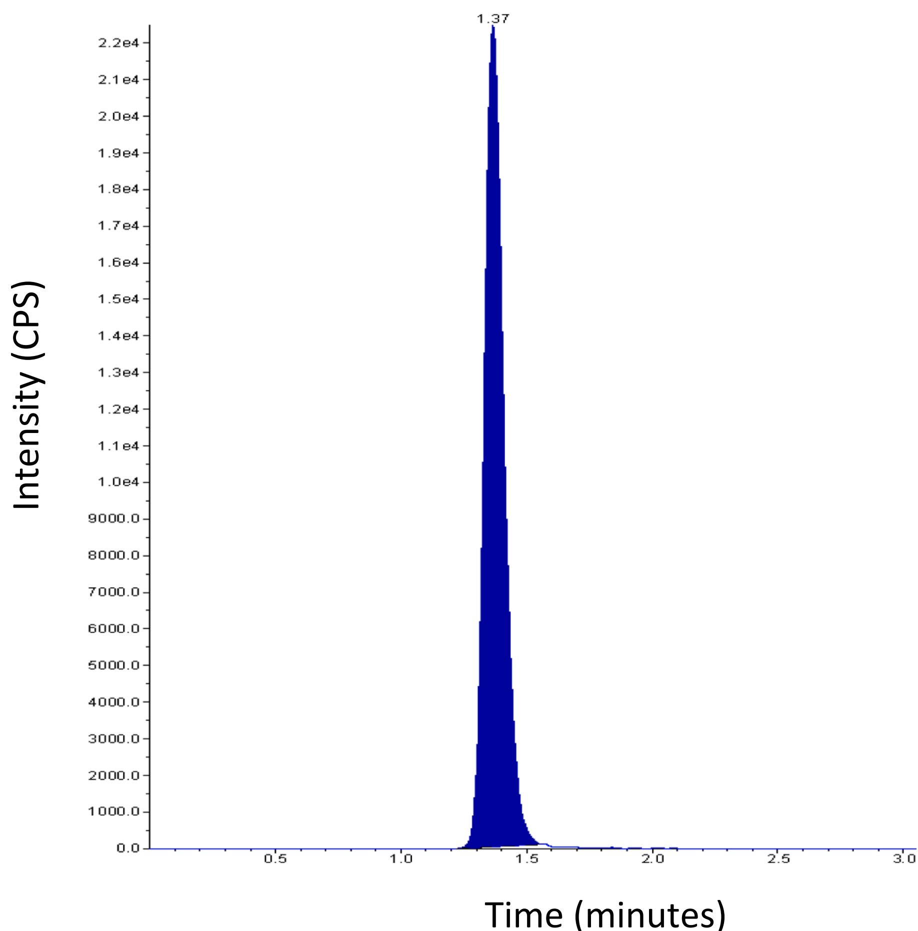

Fig. 2b. Mass Chromatograms of Internal Standard (IS).

In addition to screening blank K_2EDTA human plasma, spike-in samples were prepared in six different lots of K_2EDTA human plasma at the LLOQ level. These spike-in selectivity samples were analyzed to demonstrate the ability of the method to quantitate the analyte from different matrix lots.

For sensitivity, the LLOQ should be greater than 5 times the response of the blank sample.

The purpose of the injection carryover test was to evaluate the extent of carryover of an analyte of interest from one sample to the next in every run. An extracted blank was inserted in the injection sequence after the highest calibration standard (ULOQ) from the first and second set of standards with the same injection volume as other samples.

2.6.3. Recovery, and matrix effects

The purpose of the recovery test was to evaluate the efficiency of the liquid–liquid extraction process. Recovery was determined at three concentrations (15, 1500, and 4000 ng/mL, n = 5) for Aderamastat and at one concentration (500 ng/mL, n = 5) for the internal standard.

The recovery of the analyte in this assay was evaluated by comparing the mean peak areas from the analyte added to and recovered from the biological matrix (extracted samples) to the peak areas from the post extracted spiked samples. Recovery was calculated using the following equation:

$$\%\text{Recovery} = \frac{MeanofPeakAreasofExtractedSamples}{MeanofPeakAreasofPostExtractedSpikedSamples} \times 100$$

The purpose of the matrix effect test was to evaluate the suppression or enhancement of the analyte and internal standard responses by the matrix. The matrix effect was determined in six individual lots of K_2EDTA human plasma at two concentrations (15 and 4000 ng/mL, n = 3) for Aderamastat and at one concentration (500 ng/mL, n = 3) for the internal standard.

Matrix effect was evaluated by calculating the matrix factor for the analyte and IS using the following equations:

$$MatrixFactor = \frac{MeanofPeakAreasinthePresenceofMatrix}{MeanofPeakAreasintheAbsenceofMatrix}$$

The matrix factor was the ratio of peak areas of solutions in the presence of the matrix to the peak areas of solutions in the absence of the matrix, which serve as reference samples.

2.6.4. Stability and dilution integrity

The tests described below evaluated the stability of the analyte in situations likely to be encountered during actual sample handling and analysis.

Experiments were conducted to evaluate the stability of Aderamastat in K_2EDTA human plasma at 4 °C, through freeze/thaw cycles, and following long-term storage (−20 °C and −70 °C). Aderamastat stability was assessed in solvent (ambient temperature − 15 degrees and 25 degrees Celsius-, and –20 °C), in human whole blood, and in processed samples. Benchtop stability of Aderamastat in K_2EDTA human plasma was tested to evaluate analyte stability in the matrix at 4 °C (on wet ice) during sample handling and processing. Stability was determined at two concentrations (15 and 4000 ng/mL). The samples were stored at.

4 °C (on wet ice) for 20.5 h prior to extraction. Stability samples at two concentrations (15 and 4000 ng/mL) were frozen at −20 and −70 °C (for a minimum of 24 h for one cycle and a minimum of 12 h for the other cycles) and thawed on wet ice. After the completion of the

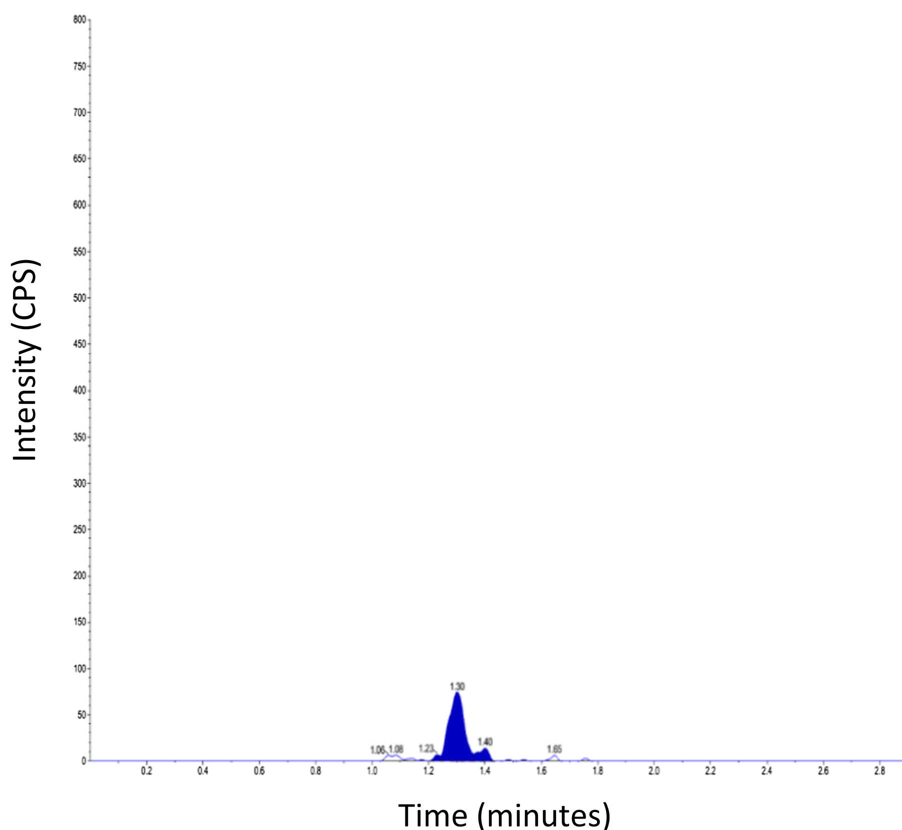

Fig. 2c. Mass Chromatograms of Blank Plasma. CPS=counts per second.

Table 4a
Blank Selectivity Test of Aderamastat (FP-025) and FP-025-d9 in K_2EDTA Human Plasma.

LotNumber	True BlankAnalyte Peak Area	% of Average Peak Area of LLOQ Standards[a]	True BlankIS Peak Area	% of Average IS Peak Area of LLOQ Standards[a]
BRH871591	ND	NC	ND	NC
BRH871593	ND	NC	ND	NC
BRH871594	ND	NC	ND	NC
BRH871595	ND	NC	ND	NC
BRH871598	ND	NC	ND	NC
BRH871600	ND	NC	ND	NC
Average Analyte Peak Area ofLLOQ Standards		1945.987	Average IS Peak Area ofLLOQ Standards	134751.426

ND=Not Detected, NC=Not Calculated, LLOQ=Lower Limit Of Quantification. IS=internal standard.
[a] % = (True Blank analyte or IS peak area/mean LLOQ standard analyte or IS peak area) x 100.

Table 4b
Spike-in Selectivity Test of Aderamastat (FP-025) and FP-025-d9 in K_2EDTA Human Plasma.

Lot Number	Spike-In Concentration 5.00 ng/mL	%RE
BRH871591	4.58	−8.4
BRH871593	4.51	−9.8
BRH871594	4.72	−5.6
BRH871595	4.50	−10.0
BRH871598	4.59	−8.2
BRH871600	4.32	−13.6
Mean	4.54	
S.D.	0.132	
%CV	2.9	
%RE	−9.2	
n	6	

SD=standard deviation, %CV=percent coefficient of variation, %RE=Percent Relative Error, n = number.

fourth cycle, the samples were analyzed. Processed sample stability was determined at 4 °C. All replicates of the low and high QC samples of a valid run were kept refrigerated prior to reinjection. Human blood that freshly collected at QPS Taiwan was pre–incubated at 37 °C for approximately 20 min. Aderamastat was spiked into pre–incubated whole blood at 15 and 4000 ng/mL. Whole blood stability solutions were inverted to mix and then incubated at 37 °C for ten minutes to reach equilibrium. After the solution reaches equilibrium, about 2.5 mL of whole blood QC samples at 15 and 4000 ng/mL, respectively, were transferred into appropriate plastic culture tubes and kept on wet ice. The plasma samples were harvested after 0, 0.5, 1, and 2 h from the blood in a refrigerated (4 °C) centrifuge for ten minutes at 3000 rpm.

Long-term storage stability was established by using freshly prepared standards that were made from spiking solutions in methanol.

A K_2EDTA human plasma sample was prepared at one concentration (10000 ng/mL) and diluted 10 folds with pooled blank K_2EDTA human plasma, each fold in five replicates to test the dilution integrity.

3. Results

3.1. Linearity, accuracy, and precision

Linear regression analysis calculations were performed with $1/x^2$ weighting using Watson™ LIMS v.7.4.1. Table 2a lists the standard curve parameters for Aderamastat and showed that, the correlation coefficients (r2) and the regression coefficients were all greater than 0.994, thus indicating a strong linear relationship for the analyzed

Table 4c
Effect of Aderamastat (FP-025) in 2% Hemolyzed K_2EDTA Human Plasma.

% Hemolysis	Concentrations (ng/mL)			
	15.0	%RE	4000	%RE
2	14.0	−6.7	3820	−4.5
	15.1	0.7	3790	−5.3
	14.5	−3.3	3650	−8.8
	14.3	−4.7	3830	−4.3
	14.4	−4.0	3760	−6.0
n	5		5	
Mean	14.5		3770	
S.D.	0.404		72.5	
%CV	2.8		1.9	
%RE	−3.3		−5.8	

SD=standard deviation, %CV=percent coefficient of variation, %RE=Percent Relative Error, n = number.

Table 4d
Effect of Aderamastat (FP-025) in Lipemic K_2EDTA Human Plasma.

Run ID	Concentrations (ng/mL)			
	15.0	%RE	4000	%RE
3	15.2	1.3	3910	−2.3
	14.7	−2.0	4020	0.5
	15.5	3.3	3970	−0.8
	15.6	4.0	4030	0.8
	15.7	4.7	4070	1.8
n	5		5	
Mean	15.3		4000	
S.D.	0.404		61.6	
%CV	2.6		1.5	
%RE	2.0		0.0	

SD=standard deviation, %CV=percent coefficient of variation, %RE=Percent Relative Error, n = number.

Table 5
Dilution Test of Aderamastat (FP-025) in a K_2EDTA Human Plasma Sample (10000 ng/mL).

Run ID	QC 10,000 (ng/mL)[a] Adjusted Concentration[b]
3	9500
	9240
	9480
	9460
	9520
n	5
Mean	9440
S.D.	114
%CV	1.2
%RE	−5.6

ID=identification, QC=quality control, SD=standard deviation, %CV=percent coefficient of variation, %RE=Percent Relative Error, n = number.
[a] Nominal Concentration.
[b] Adjusted Concentration = Determined Concentration x Dilution Factor (10-fold).

calibration curves. Table 2b lists the back–calculated calibration standard concentrations for Aderamastat in K_2EDTA human plasma. The results show a linear fit from 5 to 5000 ng/mL for Aderamastat.

The results for intraday precision and accuracy were presented in Table 3. Chromatograms of the LLOQ (5 ng/mL), IS and blank plasma are shown in Figs. 2a, 2b and 2c respectively.

3.2. Selectivity, sensitivity, and carry-over

None of the six individual lots of blank K_2EDTA human plasma showed an interference peak area at the retention time of the peak for Aderamastat that was > 20.0 % of the mean analyte peak area of the lowest calibration standards and none of the six individual lots showed an interference peak area at the retention time of the IS that was > 5.0 % of the mean internal standard peak area in the lowest calibration standards (Table 4a). For spike-in samples, the %CV of the determined concentrations from all the spiked-in selectivity samples did not exceed 20.0 %, the mean %RE and the individual %RE of the determined concentrations from all the spiked-in selectivity samples did not exceed ± 20.0 % from their nominal concentrations (Table 4b).

The mean concentration of the five hemolyzed and lipemic plasma samples replicates did not deviate more than ± 15.0 % from its nominal value. In addition, the precision (%CV) at each concentration did not exceed 15.0 % (Table 4c and Table 4d).

The carry-over effect in the blank plasma samples was less than 20 % at the LLOQ level and less than 5 % for the IS in all cases.

3.3. Dilution integrity test

The mean of the determined concentrations of the diluted samples at the given concentration was within 100 ± 15.0 % of the nominal value before dilution, and the %CV of the determined concentrations of the diluted samples did not exceed 15.0 % (Table 5).

3.4. Matrix effect & recovery

The mean IS-normalized matrix factor considered to be consistent across the validated assay range. The results have been summarized for Aderamastat in Table 6a and Table 6b.

The recovery of the analyte in this assay was evaluated by comparing the mean peak areas from the analyte added to and recovered from the biological matrix (extracted samples) to the peak areas from the post extracted spiked samples.

The results have been summarized for Aderamastat and the internal standard in (Table 7a and Table 7b), respectively.

3.5. Stability

3.5.1. Benchtop stability

The determined concentration at each level did not exceed ± 15.0 % RE from the nominal concentration, and the %CV of the determined concentrations at each level did not exceed 15.0 % (Table 8a).

3.5.1.1. Freeze/thaw stability. The determined concentrations at each level did not exceed ± 15.0 % RE from the nominal concentration, and the %CV of the determined concentrations at each level did not exceed 15.0 % (Table 8b and Table 8c).

3.5.1.2. Processed sample stability. A comparison between the QC concentrations from the reinjected run to their nominal concentrations showed that results were reproducible after storage at 4 °C for 64 h (Table 8d).

3.5.1.3. Whole blood stability. The mean peak area ratios of the stability samples at 0.5, 1, and 2 h were within 100 ± 15.0 % of the peak area ratios at Time 0. This indicate that, Aderamastat was found to be stable in K_2EDTA whole blood at 4 °C (on wet ice) for two hours (Table 8e).

3.5.1.4. Analyte stock and spiking solution stability. The stock solution of Aderamastat was stable for at least 176 days of storage in methanol at –20 °C (Table 8f). The stock solution of Aderamastat was stable for at least 23 h of storage in methanol at ambient temperature (Table 8g).

The spiking solution of Aderamastat was stable for at least 175 days of storage in methanol at −20 °C (Table 8h). The spiking solution of Aderamastat was stable for at least 23 h of storage in methanol at ambient temperature (Table 8i).

Table 6a
Matrix Effect of Aderamastat (FP-025 in K_2EDTA Human Plasma.

Concentrations (Analyte/IS)	Selectivity Lot Number					
15/500 ng/mL	BRH871591	BRH871593	BRH871594	BRH871595	BRH871598	BRH871600
	Analyte Peak Areas					
In the Presence of Matrix	6013.547	6361.479	4395.775	5443.104	5857.466	5929.908
	6463.876	6231.694	4751.947	5619.594	5636.661	5861.229
	5933.478	6560.132	4021.061	4083.305	5335.218	5998.219
n	3	3	3	3	3	3
Mean	6136.967	6384.435	4389.594	5048.668	5609.782	5929.785
In the Absence of Matrix	5828.260	5828.260	5828.260	5828.260	5828.260	5828.260
	6252.920	6252.920	6252.920	6252.920	6252.920	6252.920
	6276.055	6276.055	6276.055	6276.055	6276.055	6276.055
n	3	3	3	3	3	3
Mean	6119.078	6119.078	6119.078	6119.078	6119.078	6119.078
Matrix Factor (MF)	1.003	1.043	0.717	0.825	0.917	0.969
	IS Peak Areas					
In the Presence of Matrix (IS)	144273.327	153873.821	106764.130	133042.094	146877.290	145797.541
	156826.282	156528.205	111348.387	139420.028	135298.781	146202.131
	148377.440	156919.960	98733.313	107365.517	132026.660	149931.483
n	3	3	3	3	3	3
Mean	149825.683	155773.995	105615.277	126609.213	138067.577	147310.385
In the Absence of Matrix (IS)	144657.683	144657.683	144657.683	144657.683	144657.683	144657.683
	160228.357	160228.357	160228.357	160228.357	160228.357	160228.357
	150523.348	150523.348	150523.348	150523.348	150523.348	150523.348
n	3	3	3	3	3	3
Mean	151803.129	151803.129	151803.129	151803.129	151803.129	151803.129
IS Matrix Factor (MF)	0.987	1.026	0.696	0.834	0.910	0.970
IS Normalized MF	1.016	1.017	1.030	0.989	1.008	0.999
Mean	1.010					
S.D.	0.014					
%CV	1.4					

SD=standard deviation, %CV=percent coefficient of variation, n = numberMF=Matrix Factor, IS=Internal standard.

Table 6b
Matrix Effect of Aderamastat (FP-025) in K_2EDTA Human Plasma.

Concentrations (Analyte/IS)	Selectivity Lot Number					
4000/500 ng/mL	BRH871591	BRH871593	BRH871594	BRH871595	BRH871598	BRH871600
	Analyte Peak Areas					
In the Presence of Matrix	1115308.140	1155042.400	1569566.365	1256472.591	1223546.903	1192669.283
	1237821.118	1189398.096	1097792.895	861099.179	1194832.032	1184670.693
	1257603.502	1128533.667	1201976.657	999702.900	1222555.583	1241226.872
n	3	3	3	3	3	3
Mean	1203577.587	1157658.054	1289778.639	1039091.557	1213644.839	1206188.949
In the Absence of Matrix	933459.240	933459.240	933459.240	933459.240	933459.240	933459.240
	1107743.600	1107743.600	1107743.600	1107743.600	1107743.600	1107743.600
	668975.194	668975.194	668975.194	668975.194	668975.194	668975.194
n	3	3	3	3	3	3
Mean	903392.678	903392.678	903392.678	903392.678	903392.678	903392.678
Matrix Factor (MF)	1.332	1.281	1.428	1.150	1.343	1.335
	IS Peak Areas					
In the Presence of Matrix (IS)	119401.503	122418.320	164987.485	132042.997	128699.815	124251.081
	129095.017	127415.442	117287.619	90607.323	127732.342	125846.927
	133048.337	118202.632	128769.349	106587.780	129318.579	131589.835
n	3	3	3	3	3	3
Mean	127181.619	122678.798	137014.818	109746.033	128583.579	127229.281
In the Absence of Matrix (IS)	99104.663	99104.663	99104.663	99104.663	99104.663	99104.663
	117328.775	117328.775	117328.775	117328.775	117328.775	117328.775
	68385.386	68385.386	68385.386	68385.386	68385.386	68385.386
n	3	3	3	3	3	3
Mean	94939.608	94939.608	94939.608	94939.608	94939.608	94939.608
IS Matrix Factor (MF)	1.340	1.292	1.443	1.156	1.354	1.340
IS Normalized MF	0.994	0.991	0.990	0.995	0.992	0.996
Mean	0.993					
S.D.	0.002					
%CV	0.2					

SD=standard deviation, %CV=percent coefficient of variation, n = number, MF=Matrix factor, IS=internal standard.

3.5.1.5. IS stock solution stability. The IS stock solution of FP-025-d9 was stable for at least 172 days of storage in methanol at –20 °C (Table 8j). The IS stock solution of FP-025-d9 was stable for at least 23 h of storage in methanol at ambient temperature (Table 8k).

3.5.1.6. Long-term storage stability in matrix. Aderamastat in K_2EDTA

Table 7a
Recovery of Aderamastat (FP-025) in K_2EDTA Human Plasma.

Concentrations	15 ng/mL Peak Areas	1500 ng/mL	4000 ng/mL
Extracted	7996.005	810640.052	2129174.992
	8276.199	742084.458	2165018.167
	9272.963	702594.649	2254203.330
	8631.033	776116.070	2041159.890
	8302.975	949534.096	2080733.312
n	5	5	5
Mean	8495.835	796193.865	2134057.938
Post Extracted Spiked	9714.103	872643.215	2304920.870
	11236.426	1144517.945	2510533.048
	9838.407	854910.067	2402744.926
	10198.413	867647.346	2407484.709
	12267.821	882945.302	2452000.637
n	5	5	5
Mean	10651.034	924532.775	2415536.838
% Recovery	79.8	86.1	88.3
Mean % Recovery	84.7		
S.D.	4.4		
%CV	5.2		

SD=standard deviation, %CV=percent coefficient of variation, n = number.

Table 7b
Recovery of Internal Standard (FP-025-d9) for Aderamastat (FP-025) in K_2EDTA Human Plasma.

Concentrations(Analyte/IS)	15/500 ng/mL Peak Areas	1500/500 ng/mL	4000/500 ng/mL
Extracted	195020.449	213931.052	201127.126
	197291.600	193290.040	201900.965
	213321.532	182841.762	213764.791
	207387.806	201620.212	190128.702
	200126.512	250103.240	194581.598
n	5	5	5
Mean	202629.580	208357.261	200300.636
Post Extracted Spiked	224655.979	227301.085	218967.724
	270730.837	303606.134	234714.842
	242421.883	221751.306	222485.394
	241320.323	221598.770	228734.037
	279479.468	226764.381	231660.112
n	5	5	5
Mean	251721.698	240204.335	227312.422
% Recovery	80.5	86.7	88.1
Mean % Recovery	85.1		
S.D.	4.0		
%CV	4.7		

SD=standard deviation, %CV=percent coefficient of variation, n = number, IS=Internal standard.

Table 8a
Stability results Aderamastat (FP-025) in K_2EDTA Human Plasma: Table 8a: Wet Ice Benchtop Stability of Aderamastat (FP-025) in K_2EDTA Human Plasma.

Time (hours)	Concentrations (ng/mL)	
	15.0	4000
20.5	13.7	3970
	13.3	4000
	14.1	4070
	13.6	3990
	13.5	3980
n	5	5
Mean	13.6	4000
S.D.	0.297	39.6
%CV	2.2	1.0
%RE	−9.3	0.0

SD=standard deviation, %CV=percent coefficient of variation, %RE=Percent Relative Error, n = number.

Table 8b
Freeze/Thaw Stability of Aderamastat (FP-025) in K_2EDTA Human Plasma at −20 °C.

Cycles	Concentrations (ng/mL)	
	15.0	4000
4	12.3	3650
	12.8	3650
	12.9	3630
	14.3	3620
	13.3	3620
n	5	5
Mean	13.1	3630
S.D.	0.75	15.2
%CV	5.7	0.4
%RE	−12.7	−9.3

SD=standard deviation, %CV=percent coefficient of variation, %RE=Percent Relative Error, n = number.

Table 8c
Freeze/Thaw Stability of Aderamastat (FP-025) in K2EDTA Human Plasma at −70 °C.

Cycles	Concentrations (ng/mL)	
	15.0	4000
4	12.6	3750
	13.1	3700
	13.3	3700
	13.0	3700
	13.2	3680
n	5	5
Mean	13.0	3710
S.D.	0.27	26.1
%CV	2.1	0.7
%RE	−13.3	−7.3

SD=standard deviation, %CV=percent coefficient of variation, %RE=Percent Relative Error, n = number.

Table 8d
Processed Sample Stability of Aderamastat (FP-025) in K_2EDTA Human Plasma at 4 °C.

Time (hours)	Concentrations (ng/mL)	
	15.0	4000
64	14.1	3750
	13.3	3810
	13.4	3800
	13.8	3830
	13.3	3740
	13.2	3810
n	6	6
Mean	13.5	3790
S.D.	0.354	36.3
%CV	2.6	1.0
%RE	−10.0	−5.3

SD=standard deviation, %CV=percent coefficient of variation, %RE=Percent Relative Error, n = number.

human plasma (15 and 4000 ng/mL) was found to be stable for at least 174 days of storage at −20 °C and −70 °C (Table 8l).

4. Discussion

Here we describe the development and validation of an LC-MS/MS analytical method for rapid quantification of Aderamastat in human plasma. Stable isotope labeled Aderamastat was used as internal standard (IS) to correct for any potential matrix effect. The analytical method complied with the FDA and EMA guidelines on validating bioanalytical methods [17,19,20].

Table 8e
Stability of Aderamastat (FP-025) in K2EDTA Human Whole Blood at 4 °C (on Wet Ice).

	4 °C Peak Area Ratio			
	0-Hour	0.5-Hour	1-Hour	2-Hour
15 ng/mL	0.055884	0.055521	0.058061	0.057943
	0.056346	0.053350	0.058427	0.058630
	0.055583	0.055373	0.058204	0.057001
n	3	3	3	3
Mean	0.055938	0.054748	0.058231	0.057858
S.D.	0.000384	0.001213	0.000184	0.000818
%CV	0.7	2.2	0.3	1.4
%Diff[a]	NA	−2.1	4.1	3.4
4000 ng/mL	15.083836	15.584700	15.684934	15.342792
	15.648873	15.676943	15.432263	15.799687
	15.411042	15.398438	15.008183	15.630656
n	3	3	3	3
Mean	15.381250	15.553360	15.375127	15.591045
S.D.	0.283694	0.141873	0.341974	0.231009
%CV	1.8	0.9	2.2	1.5
%Diff[a]	NA	1.1	0.0	1.4

NA=Not applicable, SD=standard deviation, %CV=percent coefficient of variation, n = number.
[a] %Diff = ((mean of 0.5-, 1-, or 2-hour peak area ratio − mean of 0-hour peak area ratio) / mean of 0-hour peak area ratio) x 100.

Table 8f
Stock Solution Stability of Aderamastat (FP-025) in Methanol at –20 °C.

Run ID	Fresh Response[a]	Frozen Response[b]
12	4.500589	4.571530
	4.740842	4.697063
	4.768202	4.891787
	4.643359	4.623351
	4.556147	4.642605
n	5	5
Mean	4.641828	4.685267
S.D.	0.115181	0.123874
%CV	2.5	2.6
%Diff[c]		0.9

SD=standard deviation, %CV=percent coefficient of variation, n = number. ID=Identification.
[a] Peak area ratio of 100 µg/mL solution prepared from a freshly prepared stock A.
[b] Peak area ratio of a 100 µg/mL solution stored at −20 °C for 176 days.
[c] %Diff = ((mean −20 °C response − mean fresh response) / mean fresh response) × 100.

Table 8
g. Stock Solution Stability of Aderamastat (FP-025) in Methanol at Ambient Temperature.

Run ID	Reference Response[a]	Ambient Temperature Response[b]
12	4.500589	4.611155
	4.740842	4.650317
	4.768202	4.767447
	4.643359	4.689743
	4.556147	4.500552
n	5	5
Mean	4.641828	4.643843
S.D.	0.115181	0.098777
%CV	2.5	2.1
%Diff[c]		0.0

SD=standard deviation, %CV=percent coefficient of variation, n = number, ID=Identification.
[a] Peak area ratio of 100 µg/mL solution prepared from a freshly prepared stock A.
[b] Peak area ratio of a 100 µg/mL stock solution stored at ambient temperature for 23 h.
[c] %Diff = ((mean ambient temperature response − mean fresh response) / mean fresh response) × 100.

Table 8
h. Spiking Solution Stability of Aderamastat (FP-025) in Methanol at −20 °C.

Run ID	Fresh Response[a]	Frozen Response[b]
12	0.546324	0.551135
	0.580941	0.559147
	0.561788	0.593849
	0.563011	0.557507
	0.539091	0.554423
n	5	5
Mean	0.558231	0.563212
S.D.	0.016274	0.017398
%CV	2.9	3.1
%Diff[c]		0.9

SD=standard deviation, %CV=percent coefficient of variation, n = number, ID=Identification.
[a] Peak area ratio of 100 ng/mL solution prepared from a freshly prepared stock A.
[b] Peak area ratio of a 100 ng/mL solution stored at −20 °C for 175 days.
[c] %Diff = ((mean −20 °C response − mean fresh response) / mean fresh response) × 100.

Table 8i
Spiking Solution Stability of Aderamastat (FP-025) in Methanol at Ambient Temperature.

Run ID	Reference Response[a]	Ambient Temperature Response[b]
12	0.546324	0.543421
	0.580941	0.548179
	0.561788	0.557373
	0.563011	0.527170
	0.539091	0.593225
n	5	5
Mean	0.558231	0.553874
S.D.	0.016274	0.024574
%CV	2.9	4.4
%Diff[c]		−0.8

SD=standard deviation, %CV=percent coefficient of variation, n = number, ID=Identification.
[a] Peak area ratio of 100 ng/mL solution prepared from a freshly prepared stock A.
[b] Peak area ratio of a 100 ng/mL stock solution stored at ambient temperature for 23 h.
[c] %Diff = ((mean ambient temperature response − mean fresh response) / mean fresh response) × 100.

Table 8j
Internal Standard Stock Solution Stability of FP-025-d9 in Methanol at −20 °C.

Run ID	Fresh Response[a]	Frozen Response[b]
12	0.215941	0.214337
	0.213507	0.215689
	0.202240	0.215965
	0.211535	0.219705
	0.220803	0.219731
n	5	5
Mean	0.212805	0.217085
S.D.	0.006847	0.002481
%CV	3.2	1.1
%Diff[c]		2.0

SD=standard deviation, %CV=percent coefficient of variation, n = number, ID=Identification.
[a] Peak area ratio of 1000 ng/mL solution prepared from a freshly prepared stock A.
[b] Peak area ratio of 1000 ng/mL solution prepared from a stock solution stored at −20 °C for 172 days.
[c] %Diff = ((mean −20 °C response − mean fresh response) / mean fresh response) × 100.

Table 8
k. Internal Standard Stock Solution Stability of FP-025-d9 in Methanol at Ambient Temperature.

Run ID	Fresh Response[a]	Ambient Temperature Response[b]
12	0.215941	0.217974
	0.213507	0.208247
	0.202240	0.213885
	0.211535	0.219075
	0.220803	0.221234
n	5	5
Mean	0.212805	0.216083
S.D.	0.006847	0.005131
%CV	3.2	2.4
%Diff[c]		1.5

SD=standard deviation, %CV=percent coefficient of variation, n = number, ID=Identification.
[a] Peak area ratio of 1000 ng/mL solution prepared from a freshly prepared stock A.
[b] Peak area ratio of 1000 ng/mL solution prepared from a stock solution stored at ambient temperature for 23 h.
[c] %Diff = ((mean ambient temperature response − mean fresh response) / mean fresh response) × 100.

Table 8
l. Long-Term Storage Stability of Aderamastat (FP-025) in K_2EDTA Human Plasma at −20 °C and −70 °C.

Concentration (ng/mL)	Verification Run[a]	LTSS Run (174 Days at −20 °C)[b]	LTSS Run (174 Days at −70 °C)[c]
15.0	13.2	13.6	15.0
	14.0	15.2	15.4
	14.3	13.7	13.3
	12.8	14.5	14.5
	13.3	14.3	14.5
	13.7		
n	6	5	5
Mean	13.6	14.3	14.5
S.D.	0.554	0.650	0.789
%CV	4.1	4.5	5.4
%RE	−9.3	−4.7	−3.3
4000	3950	4000	4200
	4050	4240	4040
	4140	4040	4190
	4090	4210	4070
	4060	4090	4170
	4140		
n	6	5	5
Mean	4070	4120	4130
S.D.	70.8	105	73.7
%CV	1.7	2.5	1.8
%RE	1.8	3.0	3.3

SD=standard deviation, %CV=percent coefficient of variation, %RE=Percent Relative Error, n = number, LTSS=Long-Term Storage Stability.
[a] Verification Run: Run ID 1.
[b] LTSS run (174 days at −20 °C): Run ID 11.
[c] LTSS run (174 days at −70 °C): Run ID 11.

The clinical feasibility and applicability of the developed analytical method was used in 74 healthy volunteers who participated in two phase 1 clinical trials (registration numbers are: NCT02238834 and NCT03304964) and in 27 allergic asthma patients in phase 2 clinical trial (registration number: NCT03858686). Between June 2015 and December 2022, more than 1100 samples were analyzed from participants in phase 1 and phase 2 clinical trials in Taiwan and the Netherlands.

The landscape of respiratory diseases drugs is constantly developing due to the unmet need, growing patients population worldwide [21,22] and new approaches to treatment of respiratory diseases. Therefore, it is important to add the element of flexibility to the developed assay so that the analytical method is also applicable for future clinical application. The current assay is the first and the sole assay to measure Aderamastat using LC MS/MS. This method and approach was not only applied in clinical development of Aderamastat, but enables future registered Aderamastat including all formulations to be added to our assay. For linearity, a 8-point plasma standard curve was constructed, and was shown to be linear over the tested range of 5–5000 ng/ml. The mean between-run precision, within-run precision and accuracy ranged from 4.5 % to 5.5 % and a RE% from −3.5 % to −12 % respectively, for 5 ng/ml and 4000 ng/ml tested concentration.

Ideally, the prepared samples should be stable for an extended period of time, which facilitates flexibility during execution of clinical trials. For instance, different samples can be collected from several participants and/or patients, but analysis can be carried out at a later time point. The stability data of the tested concentrations showed accepted results according to the EMA and FDA guidelines on validating bioanalytical methods [23,24]. Four repeated freeze–thaw cycles had no apparent influence on the stability of plasma samples containing 15 ng/ml and 4000 ng/ml. After the fourth freeze–thaw cycle, Aderamastat plasma concentrations had deviations from the nominal values within the range of 1.9 and 370 after storage in −20 °C and 2 and 290 after storage in −70 °C, respectively. Samples containing 15 ng/ml and 4000 ng/ml were stable in wet ice after almost a day and has mean deviation of 1.4 and 0.0, respectively. Processed plasma samples were found to be stable at 4 °C for at least 64 h, allowing for several days analysis of extracted samples.

Aderamastat stability in K_2EDTA human plasma was demonstrated for four freeze/thaw cycles at −20 °C and −70 °C, 20.5 h at 4 °C, and 174 days of long-term storage at −20 °C and −70 °C. Aderamastat stability was also demonstrated in human whole blood at 4 °C for 2 h, in stock solution and spiking solution at ambient temperature for 23 h, in stock solution at −20 °C for 176 days, in spiking solution at −20 °C for 175 days, and in processed samples for 64 h at 4 °C. The quantitation range was 5 to 5000 ng/mL using a 100-μL sample volume. In addition, FP-025-d9 stability was demonstrated in stock solution at ambient temperature for 23 h and at −20 °C for 172 days.

5. Conclusion

The LC-MS/MS bioanalytical method, developed by QPS Taiwan to determine the concentration of Aderamastat in K_2EDTA human plasma, was successfully validated with respect to linearity, sensitivity, accuracy, precision, dilution, selectivity, hemolyzed plasma, lipemic plasma, batch size, recovery, matrix effect, stability and carry-over. This validated analytical method will enable clinical development of Aderamastat program and can be applied for therapeutic drug monitoring (TDM) in future clinical practice.

Funding

This study was funded by Foresee Pharmaceuticals, Taipei, Taiwan.

CRediT authorship contribution statement

Khalid S. Abd-Elaziz: Writing – original draft, Conceptualization. **Rex Cheng:** Writing – review & editing, Validation, Software, Investigation, Formal analysis, Data curation. **Joe Chen:** Writing – review & editing, Validation, Software, Investigation, Data curation. **Hans Maarse:** Writing – review & editing, Writing – original draft, Validation, Conceptualization. **Yisheng Lee:** Writing – review & editing. **Wenjin Yang:** Writing – review & editing. **Benjamin Chien:** Writing – review & editing. **Zuzana Diamant:** Writing – review & editing. **Jos Kosterink:** Writing – review & editing. **Daniël J. Touw:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

We would like to thank the lab staff who have been working on this assay.

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