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A Novel and Sensitive LC/MS/MS Method for Quantitation of Pyochelin in Human Sputum Samples from Cystic Fibrosis Patients

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Abstract

Pyoverdines and pyochelin are siderophore compounds secreted by *Pseudomonas aeruginosa* present in cystic fibrosis affected patients. The available literature on the quantification of pyoverdines and pyochelin in sputa of cystic fibrosis patients is based on detecting the fluorescence properties of the targets detecting pyoverdines and pyochelin without differentiating the types by chromagraphic separation. Within these studies pyochelin was always far less detected than pyoverdines. This is in contrast to gene expression data and suggets the pyochelin fluorescence detection method lacks sensitivity which results in the molecule being non-detectable. In this report, we describe a novel and sensitive LC/MS/MS method for quantitation of pyochelin in human sputum from cystic fibrosis affected patients with *Pseudomonas aeruginosa* (culture positive) infection. Using a validated bioanalytical method, 26 sputum samples from cystic fibrosis patients from a biobank were analyzed. Data showed the LC/MS/MS method met bioanalytical validation acceptance criteria and results of pyochelin were consistent with reported results of *Pseudomonas aeruginosa* concentration in cystic fibrosis patients. This new approach for quantitation of pyochelin in human sputum is more sensitive, reproducible and easier to reliably measure the biomarker pyochelin in cystic fibrosis patients. Monitoring pyoverdines and pyochelin in the sputum samples from cystic fibrosis patients may provide better analytical tools for cystic fibrosis new drug development.

Keywords: Biomarker; Cystic Fibrosis; Pyochelin, Pyoverdines; *Pseudomonas aeruginosa*; Quantitation; Siderophore compounds; LC/MS/MS.

Introduction

Cystic fibrosis is an inherited autosomal recessive disease which is caused by the presence of mutations in both copies of the gene for the cystic fibrosis transmembrane conductance regulator protein [1]. The lungs of patients with cystic fibrosis usually become chronically infected with *Pseudomonas aeruginosa*, a bacterium that can acquire iron by secreting two kinds of iron-chelating compounds (siderophores): pyoverdines and pyochelin (Figure 1). Siderophore-regulated iron uptake is generally considered a key factor in infections caused by *Pseudomonas aeruginosa*. The morbidity and mortality associated with cystic fibrosis is mainly related to rapid lung function decline and termed pulmonary exacerbation. There are considerable evidences that *Pseudomonas aeruginosa* is present in the lungs of the majority of cystic fibrosis patients, often in large amount i.e.>10^8 cfu/mL sputum [2].

Pyverdine group has more than 68 different peptide forms which has been characterized [3] and has been classified as three subtypes, Type I, Type II, and Type III [4]. Each subtype of pyverdine produced by individual strains is responsible for distinctive yellow-green fluorescence of certain pseudomonads and has a specific receptor for its uptake (FpvAI, FpvAII or FpvAIII, respectively) [4]. All subtypes of pyverdine have a strong ability to bind ferric ion with a formation constant of 10^{22} to 10^{35} [5] or 10^{24} to 10^{27} at pH 7.0 [6]. In mammals, most iron is bound to proteins (primarily ferritin, transferrin, lactoferrin and hemoglobin) with high affinity (Kd ~ 10^{20}) to reduce iron bioavailability to infecting bacteria. This action is an innate immunity against bacterial infection. Pyverdines are primary siderophores secreted by *Pseudomonosa aeruginosa* from infected cystic fibrosis patients to compete ferric ions from normal iron holding protein like transferrin to support the bacteria growth.

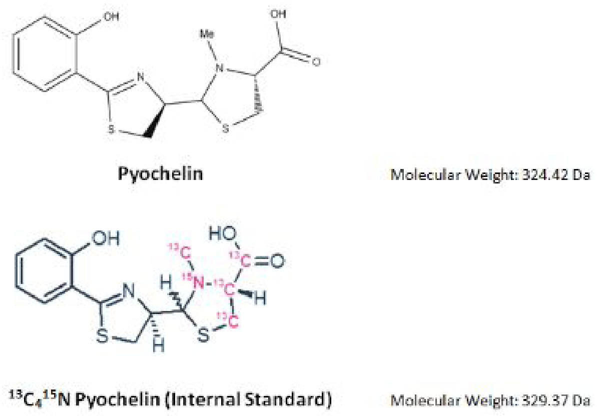

Figure 1: Chemical Structure of pyochelin and its internal Standard.

Pyochelin is considered a secondary siderophore in *Pseudomonosa aeruginosa,* having a much lower iron binding affinity than pyverdines at releasing iron from transferrin [7-9]. It has been a major focus of research on Iron acquisition by *Pseudomonas aeruginosa* related to pyverdine and pyochelin [9-13]. The analytical methods for quantitation of pyverdine and pyochelin in human sputum were based on fluorescence quenching with ferric chloride to form ferripyoverdine, yellow-greenish color at excitation 378 nm and emission 390 to 550 nm [14] or at excitation 405 nm and emission over a range of or 425-530 nm [15] for pyoverdine and at excitation 350 nm and emission 370 to 500 nm for pyochelin. These methods do not have enough sensitivity; especially pyochelin cannot be detected in cystic fibrosis patient sputa with *Pseudomonas aeruginosa* positive or low detectable concentration of [15]. There is not a pyochelin LC/MS/MS quantitation method for this type of application found in published papers. In this article, we reported a new approach to quantify pyochelin using LC/MS/MS. The method was developed and validated in a linear range of 0.25 to 25µM in human sputum. We also analyzed pyochelin concentration from 26 sputa with known *Pseudomonas aeruginosa* values from cystic fibrosis pateints and found detectable pyochelin concentrations in some of these samples.

Materials and Methods

Reagents and equipment

Pyochelin was synthesized by Sanofi R&D, Parallel Synthesis & Natural Products Chemistry, Vitry, Lot VAC.XFQ8.225.2, Molecular weight 324.42, purity 69.2%. Internal standard (IS), $^{13}C_4$-^{15}N-pyochelin (product code RA11654539, molecular weight 329.37) was synthesized by Isotope Chemistry and Metabolite Synthesis, Sanofi, Frankfurt, Germany. Acetonitrile (HPLC grade), and methanol (HPLC grade) were purchased from Burdick & Jackson (distributed by VWR Scientific Products, Newark, NJ, USA).

Ammonium acetate, PBS Buffer (10× concentrate, pH 7.4), Tween® 20, Bovine Serum Albumin (BSA), and DL-Dithiothreitol (DTT) Solution (~ 1M in H_2O) were purchased from Sigma Aldrich (St. Louis, MO, USA). Deionized water was obtained from an in-house Milli-Q DI System (Millipore, Billerica, MA, USA).

Human sputum samples collected from donors without cystic fibrosis were purchased from Bioreclamation, Westbury, New York, USA. Human sputum samples from cystic fibrosis patients with for *Pseudomonas aeruginosa* results were purchased from BioPartner, Woodland Hills, CA, USA.

The triple quadrupole mass spectrometer, model Sciex API 4000 was manufactured by Applied Biosystems (Toronto, Canada). Acquity UPLC System (H class) was manufactured by Waters Corporation (Milford, MA, USA). Multi-tube vortex was from VWR Scientific Products (Bridgeport, NJ). An HPLC column, Chromenta™ KB-SiO_2 HILIC, 2.1 mm × 100 mm, 120Å, 3 µm was purchased from Columnex LLC, San Diego, CA, USA. Centrifuge, Allegra™ 6R was manufactured by Beckman Coulter Inc. Indianapolis, IN, USA; Eppendorf micro centrifuge was made by Brinkman, Boston, MA, USA; MaxQ™ 4450 benchtop incubating orbital shaker was made by Thermo Scientific; and Sonicator was manufactured by Thomas Scientific Inc., Swedesboro, NJ, USA.

Preparation of homogenized sputum

For the matrix of pyochelin standards and quality control samples, individual human sputum samples (0.5 to 4 g, 15-20 lots from non-CF patients with *Pseudomonas aeruginosa* results) were transferred to pre-weighted 15-mL conical polypropylene tubes with cap and weighed, respectively. The net sputum weight in each tube was calculated. Using 1:1 (w/v) ratio, a volume of homogenizing solution (PBS Buffer (1×) with 0.1% (v/v) Tween® 20, 2% (w/v) BSA, 0.2% DTT) was added to each sputum sample tube to thin the viscosity of sputum. The sample tubes were vigorously vortexed and then incubated on an orbital shaker at 400 rpm at Room Temperature (RT) for 45 minutes. Then the sample tubes were vortexed briefly and centrifuged at 1000 × g for 30 minutes at RT. The top clear homogenized sputum solution (homogenized sputum) from multiple sample tubes (control sputa without detectable pyochelin) were combined, mixed into one 50-ml centrifuge tube, then the pooled homogenized sputum solution was aliquoted to 4.0 mL per tube with screw cap and stored at -80°C for future standards and QC preparation and extraction. For individual cystic fibrosis patient sputum samples, the homogenized sputum samples are prepared according to the above procedure individually (not pooled) and the homogenized sputum from each patient sample was stored at -80 °C for further extraction.

Preparation of pyochelin standards and QCs in homogenized sputum

Primary pyochelin stock solution, 2.50 mM, (0.811 mg/mL, molecular weight 324.42, with correction of purity 69.2%) was prepared in methanol. The two independently weighted pyochelin stock solutions were prepared, diluted, and chromatographically compared to be ≤ 10% difference to confirm the correctness. The two stock solutions (Stock A for calibration standards and Stock B for QCs) were then aliquoted to 100 µL per 2-ml HPLC vial for multiple vials. These HPLC vials were evaporated under nitrogen flow at 37 ºC heat block to dryness. All dry vials were stored at -20 ºc for future analysis use. At the time of sample analysis, a dry vial of pyochelin was reconstituted with 100 µL of methanol, vortexed, sonicated for 5 minutes at RT; then 900 µL of methanol was added to the same vial to make 250 µM pyochelin secondary solutions. Calibration Standards (400 -1000 µL) were prepared by mixing appropriate volume of 250 µM pyochelin stock solution A with the blank homogenized sputum to generate eight concentrations at 0.125, 0.250, 0.500, 1.25, 2.50, 5.00, 11.25, and 12.5µM which were corresponding to final sputum pyochelin concentrations of 0.250, 0.500, 1.00, 2.50, 5.00, 10.0, 22.5, and 25.0 µM due to 1:1 w/v ratio mix at homogenizing step. Quality Control (QC) samples (600 - 1500 µL) were prepared in a similar way as standards using pyochelin 250 µM stock solution B mixed with the blank sputum homogenizing supernatant to generate LLOQ, low, mid, and high QC at 0.125, 0.375, 3.75, and 10.0 µM, which were corresponding to final sputum pyochelin concentrations of 0.250, 0.750, 7.50, and 20.0 µM. Both calibration standards and QCs were prepared freshly for each run in the method validation. A system validation solution containing 0.00852 µM Pyochelin and 0.0710 µM IS in solvent of 80% Acetonitrile, 20% Water, 0.01% DTT (v/v/v) was prepared for daily system suitability check.

Extraction procedure for homogenized sputum

Aliquots of 100 µL of homogenized sputum calibration standards, QC samples, blank homogenized sputum, and unknown cystic fibrosis patient homogenized sputum samples were pipetted to pre-labelled 1.5-mL micro-centrifuge tubes. Aliquots of 1.0 mL of internal standard working solution (0.312 µM $^{13}C_4$-^{15}N-pyochelin in 100% acetonitrile) were added to each tube except the samples without internal standard (1.0 mL of IS solvent, acetonitrile used for this sample). All samples were vortexed in a multi-vortex at 2000 rpm for one minute for twice and then centrifuged in Eppendorf centrifuge at 13200 rpm for 5 minutes. The supernatant 100 µL from each tube was transferred to a 2-mL clean HPLC glass vial, then diluted with 300 µL of the diluent (80% Acetonitrile, 20% Water, 0.01% (v/v) DTT), and mixed briefly. The diluted supernatant samples (10 µL) were submitted for LC/MS/MS analysis.

LC/MS/MS Conditions

Liquid chromatographic separation procedures were carried out using a Chromenta™ KB-SiO_2 HILIC column, 2.1 mm × 100 mm, 120Å, 3 µm (Columnex LLC) at room temperature with a 5 minutes' gradient program at the flow rate of 0.4 mL/min on a Waters Acquity UPLC system with mobile phase A (MPA, 50% Acetonitrile, 50% Water, 10 mM Ammonium Acetate) and mobile phase B (MPB, 95% Acetonitrile, 5% Water, 10 mM Ammonium Acetate). The gradient program was at 0 to 3.5 minutes with a ramp 5% MPA to 50% MPA, 3.5 to 3.6 minutes from 50% to 5% MPA, and 3.5 to 5.0 minutes stayed 5% MPA. The UPLC auto sampler temperature was maintained at 4 °C. The triple quadrupole Sciex API-4000 mass spectrometer was operated at positive electrospray ionization mode in multiple reaction monitoring (MRM) mode, Ion source temperature at 550 °C, dwell time 150 milliseconds. The mass spectrometry transitions were: *m/z* 325.6→191.3 for Pyochelin and 330.2 →151.1 for IS. Both *m/z* transitions were using decluttering potential 70 volt (v), entrance potential 10v, and collision energy 35v. The collision cell exit potential was 11.0v for Pyochelin and 8.0v for IS.

Pyochelin method validation and sample analysis

According to US Food and Drug Administration bioanalytical method validation for industry guidance May 2018 [16,17], the method validation elements of specificity, sensitivity, linearity, precision and accuracy, dilution integrity, stability, carryover, extraction recovery, and matrix effect were evaluated during method validation. The software, analyst (version 1.6.1, AB Sciex, Toronto, Canada), was used for MS/MS data processing. Peak area ratios of pyochelin to internal standard were plotted versus a calibration curve. Linear regression model employing a 1/x weighting was used to calculate the concentrations in QC and unknown samples. There was not endogenous pyochelin found in pooled homogenized sputum matrix**.** Twenty-six sputum samples from BioPartners with pre-measured *Pseudomonas aeruginosa* values were individually homogenized with the homogenization buffer described in this article. The supernatant solutions of these homogenized sputum lots were extracted and analyzed on the LC/MS/MS system.

Results and Discussion

Specificity and sensitivity

The ion chromatogram of pyochelin and its internal standard from a non-CF patient sputum blank 00, a lower limit of quantitation (LLOQ) 0.25 µM pyochelin, and a CF patient sputum sample acquired in the API-4000 conditions described in the experimental section are presented in (Figure 2). The chromatograms indicated adequate signal to noise at LLOQ, being much higher than 10 fold. The method showed good specificity for pyochelin quantitation. The detection limit was 0.011 µM (3x of noise). The LLOQ of human plasma was validated 0.250 µM. The intra-and inter-day precision was expressed in terms of the coefficients of variation within a run and among runs using spiked LLOQ and QC samples. Intra-day precision of the LLOQ was from 5.0 to 10.9% for CV and intra-day accuracy (%Bias) was from 2.6 to 5.8% except the 2nd run, 21.1%. Inter-day precision and accuracy for all 4 runs were 10.4% for CV and 8.2% for bias (n =20), respectively **(**Table 1, the first column**)**. The sensitivity of the assay passed validation acceptance criteria. In our later analytical work, the API-4000 mass spectrometer was changed to Qtrap-6500 so that the assay sensitivity was able to reach to 0.030 µM which was eight time sensitive than the reported method.

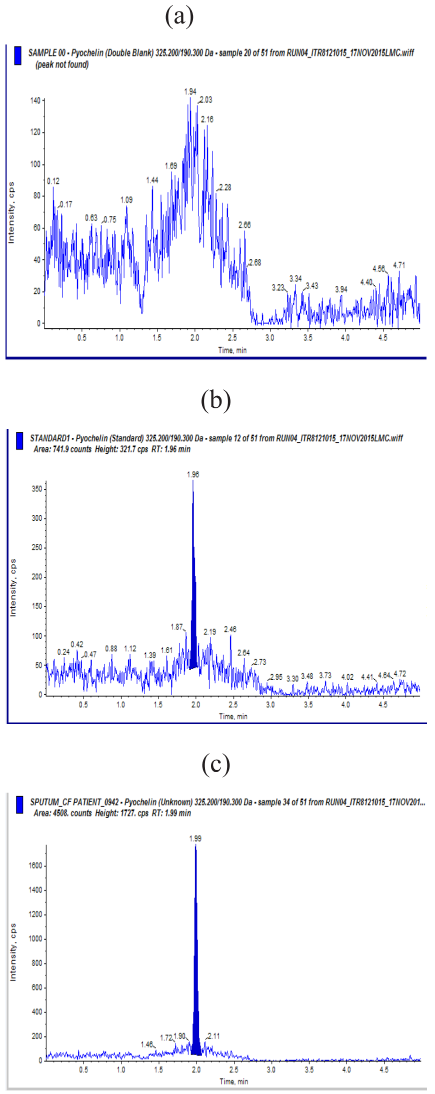

Figure 2: The ion chromatograms of pyochelin from extracted human sputum samples. (a) blank human sputum from a non-cystic fibrosis patient; (b) Spiked Pyochelin at LLOQ 0.25 µM in the blank sputum; (c) a cystic fibrosis patient sputum with pyochelin concentration at 1.33 µM.

Linearity

The pyochelin human sputum standard curve was validated from 0.25 to 25.0 µM using approximately 0.5 to 10 g of human sputum. Pyochelin concentrations (µM) were obtained using $1/x^2$ weighted linear regression analysis of the 8 calibration standards after comparison of mean absolute bias using weighted linear regression at 1, 1/x and $1/x^2$, respectively. All standard curves from the four core validation runs had coeffi¬cients of determination (r2) ≥ 0.9958. During method development, we found pyochelin calibration curve was not linear.

The concentration of DDT in final injection solution was optimized and determined that only when DDT concentration was in range of 0.01 to 0.05%, it could produce a constant response factor of pyochelin. Therefore, 0.01% DDT(v/v) was used in 300 µL of the diluent (80% Acetonitrile, 20% Water, 0.01% (v/v) DTT) for final supernatant dilution before LC/MS/MS analysis.

Precision and accuracy

The intra-day Accuracy (% Bias) QC samples at three different concentrations (0.750, 7.50 and 20.0 µM) ranged from -8.5 to 7.7% with precision (CV) ranging from 1.8 to 12.9%. Inter-day accuracy ranged from 2.8 to 6.9% for bias with a CV range from 8.0 to 11.6% **(**Table 1, the last 3 columns**)**. It was noted that the second core validation run had a bias of 21.1% for LLOQ, 21.3% for QCL, 18.1% for QCM, and 18.0% for QCL. This run should not be rejected because there was not an identified cause in operation. Our validation acceptance criteria were set as (a) at least 3 inter-day runs must pass intra-day acceptance criteria; (b) if any intra-day runs fail to meet acceptance criteria, the last two runs must consecutively pass the intra-day acceptance criteria; and (c) overall inter-day precision and accuracy for all runs must pass inter-day acceptance criteria. Therefore, overall the validation performance passed intra-day and inter-day acceptance criteria even if the 2nd run failed to meet %bias acceptance criteria.

It is worth noting that due to mixing of 1:1 weight to volume ratio of patient sputum sample to the homogenizing solution, the actual pyochelin concentration in the final homogenized sputum is only half of the patient sputum concentration. Carryover impact was performed by injecting an extracted sputum blank 00 (no Pyochelin or IS) after the highest standard (25.0 µM) in all validation runs that contained a standard curve. Carryover impact to LLOQ was calculated using the analyte or IS peak area counts in blank 00 after standard 8 divided by the analyte or IS peak area counts in Standard 1, respectively. The carryover from 7 validation runs was ≤ 7.1% for pyochelin and 0% for IS. Therefore, the carryover was acceptable for both pyochelin (≤ 20% of LLOQ) and IS (≤ 5.0% of added IS) and does not have an impact on the analytical method.

	QC ID **Conc., µM**	**LLOQ** **0.250**	**QCL** **0.750**	**QCM** **7.50**	**QCH** **20.0**
Intra-Run 4 Runs	% CV	5.0 to 10.9	2.8 to 4.7	2.5 to 12.9	1.8 to 5.1
	% Bias	2.6 to 5.8[a]	-8.5 to -0.7[b]	-4.0 to 7.7[b]	-0.3 to 1.3[b]
	n/Run	5	5	5	5
Global Inter-Run	% CV	10.4	11.6	10.2	8.0
	% Bias	8.2	2.8	6.9	5.0
	n	20	20	20	20

a) LLOQ of Runs 1, 3 and 4's intra-day and overall 4 runs inter-day met ≤ ± 20% bias acceptance criteria except the 2nd run, LLOQ's bias was 21.0%.

b) Precision and accuracy of QCL, QCM, and QCH in Runs 1, 3 and 4's intra-day and overall 4 run inter-day met ≤ ±15% acceptance criteria except the 2nd run, the bias was 21.3% for QCL, 18.1% for QCM, and 18.0% for QCL.

Table 1: Precision and Accuracy of Pyochelin in Human Sputum.

Post-extract dilution integrity

Post-extract dilution integrity was performed at dilution quality control samples (QCD2X and QCD5X, 40 µM) using the concentration of two times (2X) of the QCH level. The QCD2X and QCD5X samples were first extracted in 5 replicates per group without matrix dilution.

The final injection solution was diluted with a pool of extracted blank 0 (blank with IS) in volume of 1:1 or 1:4 of each extracted QCD2X or QCD5X sample to extracted sputum blank 0. The dilution integrity results showed that post-extract dilution had %CV from 4.0 to 5.0 % and bias ranged from -5.4 to -2.0% in Table 2. which met acceptance criteria (Table 2). This type of dilution is more convenient than the matrix dilution before sample extraction and saves time for repeat sample analysis. Sputum blank with IS (control sputum) from the same run was used as the diluent for post-extraction dilution which kept the IS concentration similar to the IS counts from standards after dilution but only the analyte concentration was diluted. In this way, pyochelin concentration in the diluted sample could be calculated from calibration curve and then multiplied by the dilution factor.

	Dilution Factor 2X QCD2X (40.0 µM)	Dilution Factor 5X QCD5X (40.0 µM)
Mean	39.2	37.8
SD	1.55	1.88
%CV	4.0	5.0
%Bias	-2.0	-5.4
n	5	5

The detailed post-extraction dilution procedures:
2X = 100 µL final sample solution mix with 100 µL of extracted blank 0 solution (with IS) and inject the sample.
5X = 100 µL final sample solution mixed with 400 µL of extracted blank 0 solution (with IS) and inject the sample.

Table 2: Dilution Integrity of Pyochelin in the Post Extracted Sample.

Stability

Stability of pyochelin was evaluated during method validation in various conditions. Results (Table 3) demonstrated that pyochelin was stable after three cycles of freeze–thaw at -80 ˚c/ room temperature and stable for up to 17 days at -80 ˚C in homogenized sputum samples. Pyochelin was stable for up to 72 hours at 4 ˚C after reinjecting extracted samples using the initial injected standard curve at time zero (or baseline) for its concentration regression. Pyochelin was also stable and reproducible after 72 hours at 4 ˚C and then reinjecting the entire run using reinjected standard curve to regress the concentration (comparing to time zero results from the regression of baseline standard curve). Pyochelin was also stable for up to 34 days at -20 ˚C in methanol (stock solution) and 24 days at -20 ˚C in 80% acetonitrile/20% water/0.01% DTT solution in the system validation solution.

Name	**Matrix**	**Conditions**	**Results**
Freeze/Thaw Stability	Homogenized Human Sputum	3 cycles at -80 ˚C/RT	%CV ≤ 6.3%, %Bias ≤ ± 10.7%
Long Term Stability	Homogenized Human Sputum	17 days at -80 ˚C	%CV ≤ 5.5%, %Bias ≤ ± 12.3%
Re-injection Stability	Diluted extracted sputum solution (82.5%ACN/ 17.5%Water/0.0075%DTT)	72 hours at 4 ˚C	%CV ≤ 3.8%, %Bias ≤ ± 5.1%
Reinjection Reproducibility	Diluted extracted sputum solution (82.5%ACN/ 17.5%Water/0.0075%DTT)	72 hours at 4 ˚C	%CV ≤ 3.7%, %Bias ≤ ± 6.5%
Stock Solution Stability	Methanol	34 days at -20 ˚C	% Difference from the fresh solution, 4.0%
SVS Stability	80%ACN/20%Water/0.01%DTT	24 days at -20 ˚C	% Difference from the fresh solution, 6.8%

Table 3: Pyochelin Stability in Homogenized Human Sputum, Processed Samples, and Solutions.

Extraction recovery

Extraction recovery was assessed by comparing the mean peak area of extracted (QCL, QCM and QCH) at each group to the mean peak area of extracted matrix blank without IS that was post-spiked with Pyochelin and IS in each group. Pyochelin or IS extraction recovery was evaluated at three concentration levels (QCL, QCM and QCH), each in five replicates (n = 5), respectively. The mean % extraction recovery, SD, and % CV at n = 5 were calculated using group mean of the peak area from extracted sample divided by mean of peak area from the post-spiked samples for pyochelin or IS, respectively. The overall mean of extraction recoveries for the analyte and IS are calculated from the three concentration group means and %CV was calculated also. The results in Table 4 show that the mean extraction recoveries were 99.2% for Pyochelin and 96.5% for the IS. The overall %CV from the three pyochelin concentration groups was ≤ 7.4% which met acceptance criteria ≤ 15%. There was not an analyte concentration dependent issue on the analyte extraction recovery. The data showed near 100% extraction recoveries for Pyochelin and IS in the sputum assay.

Validation Sample ID	**Statistics Parameters**		**Pyochelin**			**IS**	
		Pre-Spiked Peak Area	**Post-Spiked Peak Area**	**Analyte Recovery%**	**Pre-Spiked Peak Area**	**Post-Spiked Peak Area**	**IS Recovery%**
QCL 0.75 µM	Mean	3042	3065	99.2	27385	30286	90.4
	S.D.	236	172		2060	849	
	%CV	7.8	5.6		7.5	2.8	
	n	5	5		5	5	
QCM 7.50 µM	Mean	26655	26206	100.9	25471	24430	104.3
	S.D.	2842	1383		3483	1431	
	%CV	10.7	5.3		13.7	5.9	
	n	5	5		5	5	
QCH 20.0 µM	Mean	89056	91427	97.4	30971	32697	94.7
	S.D.	18052	5786		9465	2784	
	%CV	20.3	6.3		30.6	8.5	
	n	5	5		5	5	
Overall Mean Recovery				99.2			96.5
SD				1.75			7.12
%CV				1.8			7.4
n				3			3

Table 4: Extraction Recovery of Pyochelin and IS in Human Sputum.

It was observed %CV > 20% in two groups of peak area for Pyochelin and IS in QCH pre-spiked extraction group, it should not impact on pyochelin quantitation due to peak area ratio used for calculation of the analyte concentration. The stable labelled $^{13}C_4$-^{15}N-pyochelin is used as internal standard for this method, which is very important to achieve the sputum mass spectrometry assay. An analog IS (2-(4-Fluorophenyl)-4-methyl-1,3-thaiazole-5-carboxylic acid, molecular weight 237.25) was used during the method development for sample extraction in early stage of method development when stable labelled internal standard was not available. The analog internal standard showed non-parallel extraction efficiency with Pyochelin.

Sputum is not a typical secreted fluid from normal subjects' respiratory system, therefore, it can be collected from respiratory disease patients only. Also, sputum is not a homogeneous liquid so that it makes analysis of pyochelin difficult. The key procedure of the extraction method was to homogenize heterogeneous human sputum to a more uniform liquid. We developed 1:1 (w/v) ratio of sputum weight to a volume of homogenizing solution (PBS Buffer (1×) with 0.1% (v/v) Tween® 20, 2% (w/v) BSA, 0.2% DTT) to reduce the viscosity of sputum first, then vigorously vortexed and incubated samples at RT on an orbital shaker for 45 minutes to make a homogenized sputum samples. The 0.2% DDT in homogenizing solution was for breaking down thiol bonds between pyochelin or pyoverdine and various proteins in the sputum.

The 0.1%Tween 20 was used for preventing adsorption of pyoverdine from the container walls which was necessary for the ELISA assay and incorporated because the same homogenized sputum sample was used for multiple bioanalyses and shared by different sites. Fortunately, due to the large dilution in our LC/MS/MS assay extraction, the tween 20 (the suppression effect for mass spectrometer ionization) did not impact the LC/MS/MS quantitation of the analyte. The 2% BSA was for a better homogenization of sputum to a solution. After the sample tubes were vortexed and centrifuged, the supernatant of the homogenized sputum sample was stored at -80 °C for further protein precipitation extraction and LC/MS/MS analysis. During method development, we found that it was unsuccessful to use artificial sputum to prepare standard curve, because the extracted artificial sputum had a strong chromatographic interference and high viscosity so that the analytical column pressure was too high to operate. The reported analytical homogenization and extraction methods worked well for pyochelin quantitation without sacrificing LC pressure condition.

Matrix effect

Absolute matrix effect was assessed by comparing the peak area counts of post-extracted blank sputum to neat solution. Both post-extracted sputum and neat solution samples (100µL) were spiked with Pyochelin and IS (300 µL of 0.0114 µM pyochelin and 0.0946 µM IS for QCL, 0.114 µM pyochelin and 0.0946 µM IS for QCM, and 0.303 µM pyochelin and 0.0946 µM IS for QCH) to reach theoretical concentration of extracted QCL, QCM or QCH in the solvent of 80% Acetonitrile, 20% Water, 0.01% DTT. The peak area counts of Pyochelin and IS in the corresponding post-extracted sputum and neat solutions were evaluated for matrix effect. Absolute Matrix Effect (%) is calculated by subtracting mean neat solution peak area from the post-spiked QC peak area, then dividing by mean neat solution peak area, and finally multiplying 100. Table 5 showed that matrix effect was -5.6% for Pyochelin and -5.9 % for IS. The results indicate a minor ion suppression for both pyochelin and IS during mass spectrometry analysis. However, the level of suppression for Pyochelin and its stable labelled IS was consistent. Use of peak area ratio for quantitation compensates this suppression effect. The relative matrix effect assessment across multiple sputa was not evaluated in this method validation due to the rare matrix of sputa from cystic fibrosis patients.

Sample Name		Pyochelin				IS		
	Post-spiked Peak Area	Neat Solution Peak Area	Mean Neat Solution Peak Area	% Matrix Effect	Post-spiked Peak Area	Neat Solution Peak Area	Mean Neat Solution Peak Area	% Matrix Effect
QCL (0.750 µM)	10930	11690	11708	-6.6	80590	88650	86678	-7.0
	11730	11860		0.2	84910	86860		-2.0
	10950	12020		-6.5	83260	87830		-3.9
	11100	12420		-5.2	79640	87190		-8.1
	11840	10550		1.1	83360	82860		-3.8

QCM (7.50 μM)	103000	109700	114200	-9.8	82350	86720	88896	-7.4
	105900	118200		-7.3	83700	86310		-5.8
	106900	114200		-6.4	84040	88640		-5.5
	105000	111200		-8.1	81090	91120		-8.8
	107800	117700		-5.6	82070	91690		-7.7
QCH (20.0 μM)	289700	305200	312420	-7.3	88070	96000	95166	-7.5
	297600	308300		-4.7	92500	92170		-2.8
	296400	306600		-5.1	88670	95160		-6.8
	290100	316700		-7.1	88660	95210		-6.8
	296400	325300		-5.1	90300	97290		-5.1
%Overall Mean Matrix Effect				**-5.6**				**-5.9**

Table 5: Matrix Effect of Pyochelin and IS in Extracted Human Sputum.

Clinical application

Using this reported analytical method, the concentrations of pyochelin from 26 cystic fibrosis patient samples were analyzed. The results are listed in Table 6. All 26 cystic fibrosis patient sputa from BioPartners Inc were cultured for *Pseudomonas aeruginosa* and other microbiology organism bacteria counts by the vender before the samples were received at our site. These bacteria counts are also reported with pyochelin results. There were 9 out of 26 sputum samples with detectable Pyochelin concentrations, among the 9 sputum lots, 5 sputum lots were above LLOQ using the validated method. The chromatograms of quantified pyochelin and detected pyochelin are showed in Figure 2. Our method was subsequently transferred to another site and partially validated using Qtrap-6500 mass spectrometer. The assay LLOQ was lowered to 30 nM which was 8.3 folds lower than reported LLOQ (0.250 µM) in this article. Using the more sensitive mass spectrometer, the pyochelin concentration was quantified in sputa from cystic fibrosis patients (additional set of cystic fibrosis patient samples). These results were consistent with pyoverdine concentrations tested in the same sputa with *Pseudomonas aeruginosa* culture positive results (data not shown here).

Sample #	Sample Name	Conc. (µM)	**Comments**
1	SPUTUM_CF PATIENT_0910	BQL	NN - solitary; EA 9×10^{4}cfu/ml; PA 5×10^{2}cfu/ml; SA 5×10^{3}cfu/ml; CA 1×10^{4}cfu/ml
2	SPUTUM_CF PATIENT_0911	BQL	NN - solitary; SA 5×10^{5}cfu/ml
3	SPUTUM_CF PATIENT_0912	BQL	PA 1×10^{5}cfu/ml; SA 1×10^{8}cfu/ml
4	SPUTUM_CF PATIENT_0913	BQL	NN - solitary; SE - solitary; CA 5×10 cfu/ml
5	SPUTUM_CF PATIENT_0916	BQL	SA 1×10^{4}cfu/ml; BHS (not group A) 1×10^{3}cfu/ml
6	SPUTUM_CF PATIENT_0918	BQL(0.110*)	PA 1×10^{8}cfu/ml
7	SPUTUM_CF PATIENT_0919	BQL (0.209*)	NN - solitary; SVs - solitary; PA 5×10^{2}cfu/ml; SA 1×10^{3}cfu/ml; CA 5×10^{2}cfu/ml
8	SPUTUM_CF PATIENT_0921	0.284	PA 1×10^{8}cfu/ml; SA 5×10^{4}cfu/ml
9	SPUTUM_CF PATIENT_0922	0.324	PA 5×10^{6}cfu/ml; SA 1×10^{8}cfu/ml

10	SPUTUM_CF PATIENT_0935	BQL	PA 2×10^2cfu/ml; SAs 1×10^4cfu/ml
11	SPUTUM_CF PATIENT_0936	BQL	SAH 1×10^5 cfu/ml; CA 1×10^4 cfu/ml
12	SPUTUM_CF PATIENT_0939	BQL (0.171*)	PA; SA
13	SPUTUM_CF PATIENT_0941	BQL	PA 1×10^8 cfu/ml; SA 1×10^6 cfu/ml
14	SPUTUM_CF PATIENT_0942	1.33	PA 1×10^8 cfu/ml; SA 1×10^8 cfu/ml
15	SPUTUM_CF PATIENT_0945	BQL(0.031*)	PA 10^8 cfu/ml
16	SPUTUM_CF PATIENT_0946	BQL	CA and other Candida.nonalbicans 10^6 cfu/ml; SA 10^8 cfu/ml
17	SPUTUM_CF PATIENT_0947	BQL	PA 1×10^6 cfu/ml; SA 10^8 cfu/ml
18	SPUTUM_CF PATIENT_0948	BQL	PA 1×10^8cfu/ml;SA 1×10^8cfu/ml
19	SPUTUM_CF PATIENT_0954	0.390	PA1×10^6cfu/ml; CA 5×10 cfu/ml
20	SPUTUM_CF PATIENT_0955	0.294	PA 1×10^5cfu/ml
21	SPUTUM_CF PATIENT_0956	BQL	PA 5×10^5cfu/ml; CA 5×10^2cfu/ml;EF 5×10^3cfu/ml;
22	SPUTUM_CF PATIENT_0957	BQL	PA 5×10^3cfu/ml; CA 1×10^2cfu/ml; SA 1×10^3cfu/ml; EF 1×10^5cfu/ml
23	SPUTUM_CF PATIENT_0960	BQL	PA 5×10^5cfu/ml; SA1×10^4cfu/ml
24	SPUTUM_CF PATIENT_0970	BQL	SA 5×10^3cfu/ml; SV
25	SPUTUM_CF PATIENT_0971	BQL	CA 1×10 cfu/ml; EC 1×10^3cfu/ml; SA 5×10^3cfu/ml
26	SPUTUM_CF PATIENT_0973	BQL	PA 5×10^3 cfu/ml; SA 5×10^3 cfu/ml

Note: *Concentration level is below quantitation limit (≤ 0.250 µM, BQL).

BHS: *Beta-hemolytic streptococcus;* CA: *Candida albicans*; EC: *Enterobacter cloacae* ; EF: *Enterococcus faecium;* NN: *Nonpathogenic neisseria* ; PA: *Pseudomonas aeruginosa;* SA: *Staphylococcus aureus;* SAH: *Streptococcus a-haemolyticus;* SE: *Staphylococcus epidermidis;* SV: *Streptococcus viridans.*

Table 6: Pyochelin Concentration in Sputa of Cystic Fibrosis Patients.

Conclusions

A novel and sensitive analytical approach for quantitation of pyochelin in sputa from cystic fibrosis patients was developed and validated using LC/MS/MS. The method was demonstrated to be accurate and reproducible for use in a clinical setting. The performance characteristics of the assay met the bioanalytical method acceptance criteria. Using the reported method, the concentrations of pyochelin from cystic fibrosis patients were analysed. Quantifiable pyochelin concentrations in these cystic fibrosis patient sputa also had high corresponding *Pseudomonas aeruginosa* culture results. However, given the method sensitivity, high positive *Pseudomonas aeruginosa* results are not a prerequisite to find detectable pyochelin. Per our knowledge, this is the first mass spectrometry quantitation method for pyochelin in human sputum. This LC/MS/MS assay provides a new and sensitive approach to quantify pyochelin in human sputum and its use as a biomarker may enhance new drug development for cystic fibrosis.

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Conflicts of Interest

There is not a conflict of interest reported by authors. The work was supported by Sanofi Research and Development funding.

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