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Determination of Pirfenidone and Metabolites in Rat Plasma by Coupling On-Line Fractionation with LC-MS/MS

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OVERVIEW

PURPOSE

To determine concentrations of Pirfenidone (PFD) and its three metabolites, 4'-hydroxy-PFD (OH-PFD), 5-hydroxymethyl-PFD (CH_2OH-PFD) and 5-carboxy-PFD (COOH-PFD) in rat plasma using a single assay coupling LC-MS/MS with on-line trapping and fractionation.

METHODS

PFD and metabolites were extracted from rat plasma by protein precipitation and injected for on-line trapping (Acquity BEH C_{18}). Elution from the trapping column was divided into two fractions via multiple valve switching. The initial fraction containing CH_2OH-PFD/COOH-PFD was eluted with an isocratic mobile phase containing 10% MeOH. The trapping column was next switched off-line and CH_2OH-PFD and COOH-PFD were separated using a MeOH gradient from 10 to 20%, after which a second fraction containing OH-PFD/PFD was eluted from the trapping column with subsequent isocratic separation using 20% MeOH. Analytes were detected in positive ESI-MRM mode using a SCIEX 6500$^+$ TripleQuad with duty cycle optimized by period splitting.

RESULTS

Chromatographic separation capacity was achieved by an orthogonal trapping approach allowing optimization of the MS duty cycle with > 100 ms per MRM transition, resulting in S/N > 10:1 for all analytes. Further, the organic content could be optimized to allow greater ionization efficiency for OH-PFD, whose detection limits were 10-fold lower than PFD/COOH-PFD. In addition, transfer to the analytical phase with 10% MeOH allowed resolution of CH_2OH-PFD from an ion suppressor that could not be eliminated using gradient elution on a single analytical column alone (i.e. orthogonal phases were required for separation). Sample run time could be kept to only 2.5 min, including in-line flushing of both trapping and analytical columns after each injection with 100% MeOH.

Assay evaluation included determination of precision and accuracy, specificity, matrix effect and matrix factor, with all acceptance criteria being met. A single assay was therefore feasible for the quantitation of all four analytes extracted from rat plasma whose dynamic range differed 10-fold.

INTRODUCTION

Pirfenidone (PFD) is a medication approved for the treatment of idiopathic pulmonary fibrosis. The scope of the current research involved quantitation of PFD and its three metabolites, 5-carboxy-PFD (COOH-PFD), 5-hydroxymethyl-PFD (CH_2OH-PFD) and 4'-hydroxy-PFD (OH-PFD), in a single assay. The challenge of developing LC-MS/MS methodology originated from the disparate polarity of the four analytes and their 10-fold difference in LOQ, requiring careful titration of chromatographic separation, linearity, carryover, MS duty cycle and progeny ion selection. Therefore, on-line fractionation was adopted, allowing adequate analyte separation for period splitting whilst minimizing carryover, maintaining linearity of response, and eliminating ion suppression for CH_2OH-PFD.

METHOD

SAMPLE PREPARATION

An aliquot of 10 µL rat plasma was extracted by adding 240 µL of MeOH, which contained stable labeled internal standards (PDF-D3, OH-PDF-D3, CH_2OH-PFD-D5 and COOH-PFD-D5). Following centrifugation, supernatants (30 µL) were further diluted with 210 µL of 0.02% HCOOH. Extracts were stored at 4 ºC for LC-MS/MS analysis.

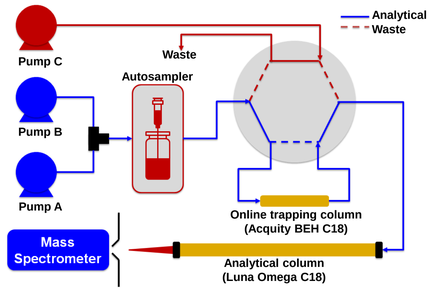

Figure 1. System Configuration for On-line Fractionation

LC-MS/MS

Chromatographic separation was performed using a Shimadzu LC-30AD NexeraX2 UPLC system including three isocratic pumps and one 6-port valve for on-line trapping (**Figure 1**). Conditions were as follows:

- On-line trapping column: Acquity BEH C18, 5 x 2.1 mm, 1.7 µm
- Analytical column: Luna Omega Polar C18, 50 x 2.1 mm, 1.6 µm
- Mobile phase (Pump A/B): 0.02% HCOOH/MeOH
- Flow Rate: 1.0 mL/min
- Column Temperature: 30 ^{0}C
- Flush solution for on-line trapping column (Pump C): 1.0 mM oxalic acid, 0.1% HCOOH in H_2O:ACN:MeOH (2:1:1)
- SCIEX API 6500$^+$, MRM acquisition in positive ion ESI (**Table 1**)

Table 1. MRM Transitions and Collision Energies

Analyte	Q1	Q3	CE
PDF	186.1	158.1	45
PDF-D3	189.1	161.1	45
OH-PDF	202.1	92.1	35
OH-PDF-D3	205.1	95.1	35
CH_2OH-PFD	202.1	126.1	38
CH_2OH-PFD-D5	207.1	131.1	38
COOH-PFD	216.1	122.1	50
COOH-PFD-D5	221.1	122.1	50

RESULTS

ON-LINE FRACTIONATION

The establishment of on-line fraction required sufficient separation resolutions for these target analytes on the trapping column. **Figure 2** showed the typical chromatogram of PDF and its metabolites on Acquity BEH C18 guard column (5 mm x 2.1 mm *i.d.*). It was indicated that these four analytes could be divided into two groups, based on their polarities and reversed-phase (RP) retentions. Thus, a step-elution procedure could be implemented to transfer the analytes into the analytical column with multiple mobile phase conditions (**Table 2**).

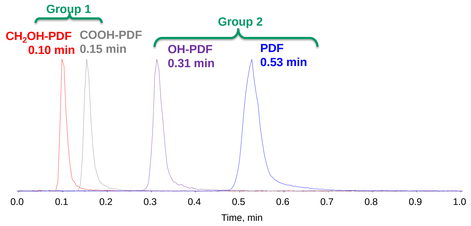

Figure 2. Chromatogram of PDF, OH-PFD, CH_2OH-PFD and COOH-PFD on Acquity BEH C18 guard column (on-line trapping column) with the mobile phase composited of 0.02% HCOOH:MeOH, 90:10% v/v at a flow rate of 1.0 mL/min

SEPARATION

Retention for CH_2OH-PFD/COOH-PFD required low MeOH content (~10%) whilst avoidance of peak shape distortion for OH-PFD/PFD and reasonable throughput required a MeOH content ≥ 20% (**Figure 3A** and **3B**). Although a gradient separation for all four analytes in a single injection was feasible, column carryover for highly retained OH-PFD/PFD was noted, requiring complicated column flushing and prolonged run time.

Table 2. Time Program for On-line Fractionation

Time (min)	Trapping Column	% MeOH	Note
0.00	Analytical	10%	Sample injected
0.20	Waste	10%	Trapping column off-line, without flow
0.60	Waste	20%	MeOH content increased for separation of CH_2OH-PDF / COOH-PDF and for 2nd elution
1.00	Analytical	20%	2nd elution from trapping column
1.20	Waste	20%	Trapping column off-line, without flow
2.00	Analytical	20%	Separation of analytes
2.01	Analytical	10%	Equilibration of trapping and analytical column
2.50	Analytical	10%	Run ended

RESULTS (CONT.)

The implementation of on-line fractionation involved a two-step transfer from trapping to analytical column. All four analytes were eluted at optimal mobile phase conditions which mitigated on-column carryover, increased background noise and peak asymmetry (**Figure 3C**).

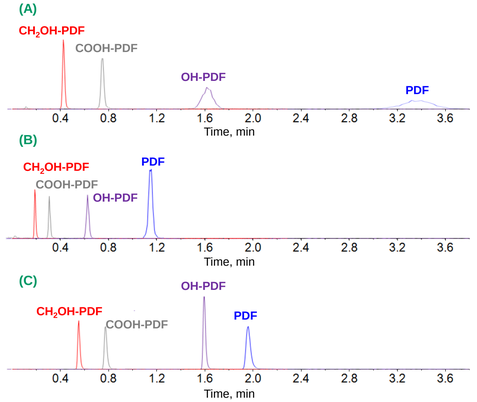

Figure 3. Chromatogram of PDF, OH-PFD, CH_2OH-PFD and COOH-PFD (A) without on-line trapping and with an isocratic mobile phase containing 10% MeOH; (B) 20% MeOH and (C) with on-line trapping and step elution.

The LC separation capacity afforded by an orthogonal trapping approach allowed dwell times of ca.100 ms, resulting in S/N > 10:1 for all analytes. Further, the organic content could be optimized to allow greater ionization efficiency for OH-PFD, whose detection limits were 10-fold lower than PFD/COOH-PFD. In addition, transfer to the analytical phase with 10% MeOH allowed resolution of CH_2OH-PFD from an ion suppressor that could not be eliminated using gradient elution on a single analytical column alone (i.e. orthogonal phases were required for separation). Sample run time could be kept to only 2.5 min, which included in-line flushing of both trapping and analytical columns with 100% MeOH after each injection.

METHOD EVALUATION

Method evaluation for all four analytes included an assessment of linearity, precision and accuracy, specificity, matrix effect and matrix factor. Results demonstrated good sensitivity and linearity (1/x^2) for the concentration ranges 20.0 - 20,000 ng/mL (PDF/COOH-PDF) and 2.00 – 2,000 ng/mL (OH-PDF/CH_2OH-PDF). Representative extracted LLOQ chromatograms are shown in **Figure 4**, and within-run precision and accuracy in **Table 4**.

RESULTS (CONT.)

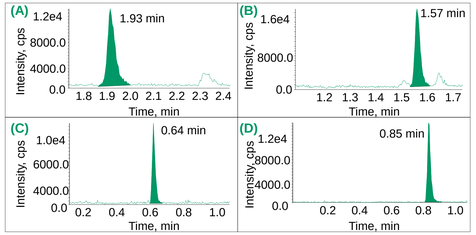

Figure 4. Chromatogram of an extracted LLOQ from rat plasma (A) 20.0 ng/mL PDF, (B) 2.0 ng/mL OH-PFD, (C) 2.0 ng/mL CH_2OH-PFD and (D) 20.0 ng/mL COOH-PFD.

There were no significant interferences in six lots of rat plasma including one lot with 5% hemolysis, indicating 100% specificity for analytes and their respective stable labeled internal standards. Matrix effect was determined from the same donors at both low and high QC concentrations, with all acceptance criteria being met.

Table 4. Within-run Precision and Accuracy for PDF and Metabolites

	QC LOQ	Low QC	Mid QC	High QC
Analyte		PDF		
Conc. (ng/mL)	20.0	60.0	1000.00	15000.00
%C.V.	1.9	3.4	2.1	2.2
%Nominal	96.3	97.6	98.8	106.2
Analyte		OH-PDF		
Conc. (ng/mL)	2.00	6.00	100.00	1500.00
%C.V.	9.9	4.1	2.4	1.8
%Nominal	108.5	99.7	97.2	102.0
Analyte		CH_2OH-PDF		
Conc. (ng/mL)	2.00	6.00	100.00	1500.00
%C.V.	3.8	2.0	2.3	2.3
%Nominal	95.5	97.5	104.3	103.0
Analyte		COOH-PDF		
Conc. (ng/mL)	20.0	60.0	1000.0	15000.0
%C.V.	3.3	1.5	2.2	1.6
%Nominal	98.8	101.9	100.1	104.0

CONCLUSION

An orthogonal on-line fractionation approach was leveraged for the determination of PFD and metabolites in rat plasma using a single assay, overcoming challenges associated with limited sample volume and disparate analyte polarity and concentration ranges.

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