



A Rapid Approach to Resolve a Matrix Effect in Complex Mixture of Analytes

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INTRODUCTION

Pre-clinical and Phase I clinical studies have revealed that OPC-P readily metabolizes to form at least eleven known metabolites. A method was needed to quantitate each metabolite because the potential activity of each known metabolite is still under investigation. However, because the parent drug and metabolites span a wide range of polarities, the challenge of developing a single assay to quantitate all of the compounds is increased.

This paper presents an approach used during the initial and advanced method development stages that allowed a rapid resolution to matrix effects that affected some analytes during inter-lot matrix testing.

METHOD DEVELOPMENT OBJECTIVES

- To develop a quantitative method for the parent and ten of the known metabolites
- Based on shared characteristics among the analytes, identify SPE and HPLC conditions to produce acceptable responses for all analytes
- Simultaneously evaluate the optimal conditions and potential alternatives for each individual analyte
- Stress method performance parameters to isolate and resolve any potential problems
- Incrementally modify the method, record major performance changes and reevaluate until desired performance level is achieved

EXPERIMENTAL

SPE phases:

- IST C₂, C₈, C₁₈, multimode, H₂C, C₁₈ (Jones Chromatography, Lakewood, CO)
- Waters Oasis HLB (Waters Corporation, Milford, MA)

HPLC columns (2 mm x 50 mm):

- Luna Phenyl/Hexyl (Phenomenex, Torrance, CA)
- Asahipak ODP (Keystone Scientific, Bellefonte, PA)
- Lightning C₈ (Jones Chromatography, Lakewood, CO)

SPE Procedure:

- 200 μ L of acidified human plasma were applied to preconditioned SPE cartridges
- The SPE cartridges were washed with aqueous buffer

- After drying, the analytes were eluted from the cartridge with methanol
- Extracts were evaporated to dryness and reconstituted in 150 μ L of acetonitrile/water

LC-API/MS/MS:

- Injection Volume: 3 μ L
- LC Program: 12 minute gradient; 16 minute cycle
- Ionization Mode: Positive ion
- Scan Type: Multiple reaction monitoring (MRM)

Instrumentation:

- LC10-ADvp pumps, Shimadzu (Columbia, MD)
- PE200 Autosampler Perkin Elmer (Norwalk, CT)
- API 3000 mass spectrometers PE Sciex (Concord, Ontario)

INITIAL METHOD DEVELOPMENT

Significant Points:

1. Based on the isotopic contributions possible (Table 1), chromatographic separation among many of the analytes was required.
2. Several common structural characteristics between the parent and metabolites (multiple aromatic rings, amine functionalities, and a high degree of hydrophobicity) were identified and used to determine the initial SPE and HPLC conditions.
3. The three best HPLC columns for resolution, symmetry, intensity and reproducibility were determined experimentally (i.e., phenyl/hexyl, C₈ and ODP). Initial method development SPE samples were injected on each HPLC column. (Figures 1-3).
4. The phenyl/hexyl column produced chromatograms with excellent peak shape and sensitivity and demonstrated superior resolving power. It achieved baseline separation for many of the analytes (Figure 1).
5. Nonpolar SPE phases (C₁₈, C₈, and universal polymers) retained the analytes adequately, but also retained and eluted more exogenous material resulting in greater variability.
6. The C₈ and ODP HPLC columns possessed less resolving power and sensitivity, but both columns were still very acceptable.
7. The C₂ SPE extraction with phenyl/hexyl HPLC column was determined experimentally to provide the optimum precisions and recoveries.

ADVANCED METHOD DEVELOPMENT

Identify and isolate potential problems

- Analyte response was evaluated among different lots of control matrix.
- All analytes showed acceptable precision within each lot of matrix as shown in Table 2 (four analytes shown).
- The response of the parent compound (OPC-P) in extracted samples varied significantly between matrix lots indicating a matrix effect (Table 2).

TROUBLESHOOTING APPROACH

The two options available to overcome the problem that was identified were either the SPE conditions or mode, or modify the HPLC conditions.

Accurate records were maintained of each method development experiment performed. Particular care was taken because of the number of analytes and the difficulties expected.

Because of the initial investigations between SPE phases and HPLC columns, it was quickly established that the SPE conditions were optimal for the analytes and the greatest selectivity gain would be achieved by modifying the HPLC conditions.

Blank plasma samples from different lots were spiked with the analytes after the C₂ extraction (post-extract). These were then injected on the three HPLC columns.

RESULTS

1. The post-extract spiked sample results are shown in Table 3. The OPC-P inter-lot variability decreased significantly for both the Asahipak ODP and Lightning C₈ columns.
2. The peak area ratios for the metabolites vary ~6% from the overall mean while peak area ratios for OPC-P varied up to 24% among the plasma lots (Table 3).
3. Two metabolites demonstrated increased variability and slight matrix effect using the Asahipak ODP column.
4. The Lightning C₈ column demonstrated acceptable resolution for the interferent and OPC-P with minimal or no apparent affect on the other analytes.

CONCLUSIONS

- During initial method development, characterize multiple SPE / HPLC phases and document the results.
- Evaluate multiple lots of control matrix during development to aid in identifying and isolating any potential problems
- Modify the variable that will have the greatest impact on the problem but will have virtually no impact on the remaining portion of the assay.
- The dynamic range of the assay was validated from 0.500 to 250 ng/mL for all analytes and is being used to quantitate these analytes in clinical trials.

Table 1. Peak Area Ratio Summary of Monitored Ions and Problematic Ions

NACL ID	R.T.	Precursor	Product	Cl isotope	-HOH
OPC-M19	3:49	458.2	212	460.2	440.2
OPC-M3	4:20	486.2	240	488.2	
OPC-M11	4:53	416.2	154	418.2	398.2
OPC-M23	5:08	416.2	154	418.2	398.2
OPC-M7	5:28	486.2	208	488.2	
OPC-IS2	5:30	366.2	120	368.2	
OPC-M5	6:14	430.2	208	432.2	412.2
OPC-M4	6:19	400.2	154	402.2	
OPC-M6	6:49	468.2	222	470.2	
OPC-M10	7:39	470.2	208	472.2	452.2
OPC-M16	7:41	412.2	208	414.2	
OPC-IS1	7:46	399.2	238	401.2	
OPC-P	9:18	454.2	208	456.2	
OPC-Mu	---	468.2	208	470.2	

Table 2. Peak Area Ratio Summary Results for Parent and Three Metabolites

Analyte	OPC-M3				OPC-M4			
	1	2	3	4	1	2	3	4
Lot Number								
Replicate								
1	0.0551	0.0588	0.0580	0.0550	0.2621	0.2812	0.2670	0.2742
2	0.0604	0.0656	0.0624	0.0615	0.2360	0.2503	0.2249	0.1993
3	0.0566	0.0653	0.0700	0.0631	0.2001	0.2275	0.2463	0.2522
Overall Mean	0.0610				0.2434			
Average	0.0574	0.0632	0.0635	0.0598	0.2328	0.2530	0.2461	0.2419
Standard Deviation	0.0027	0.0039	0.0060	0.0043	0.0312	0.0270	0.0210	0.0385
% Deviation from Overall Mean	-5.9%	3.7%	4.1%	-1.9%	-4.4%	3.9%	1.1%	-0.6%
% RSD	4.8%	6.1%	9.5%	7.2%	13.4%	10.7%	8.6%	15.9%

Analyte	OPC-M16				OPC-P			
	1	2	3	4	1	2	3	4
Lot Number								
Replicate								
1	0.5633	0.5690	0.6113	0.5630	0.9414	1.667	1.403	2.004
2	0.6265	0.6946	0.6355	0.6168	1.001	1.480	1.102	1.393
3	0.6147	0.6787	0.6365	0.6338	1.007	1.458	1.176	1.350
Overall Mean	0.6203				1.332			
Average	0.6015	0.6474	0.6278	0.6046	0.9832	1.535	1.227	1.582
Standard Deviation	0.0336	0.0684	0.0143	0.0370	0.0363	0.1148	0.1569	0.3657
% Deviation from Overall Mean	-3.0%	4.4%	1.2%	-2.5%	-26.2%	15.2%	-7.9%	18.8%
% RSD	5.6%	10.6%	2.3%	6.1%	3.7%	7.5%	12.8%	23.1%

Table 3. Peak Area Ratio Summary Results for Post-extract Spiked Parent Using Multiple Plasma Lots and Three HPLC Columns

C₂ SPE & Luna Phenyl-Hexyl HPLC

Analyte	OPC-P				
	1	2	3	4	5
Lot Number					
Replicate					
1	1.556	0.9545	1.692	2.145	2.058
2	1.453	1.138	1.666	2.057	2.076
3	1.639	1.104	1.765	1.869	2.061
Overall Mean	1.682				
Average	1.550	1.065	1.708	2.024	2.065
Standard Deviation	0.0930	0.0975	0.0515	0.1409	0.0096
%Deviation from Overall Mean	-7.9%	-36.7%	1.5%	20.3%	22.7%
%RSD	6.0%	9.1%	3.0%	7.0%	0.5%

C₂ SPE & Asahipak ODP HPLC

Analyte	OPC-P			
Lot Number	1	2	3	4
Replicate				
1	0.848	0.8629	0.944	0.902
2	0.929	0.969	0.842	0.764
3	0.928	0.909	0.856	0.850
Overall Mean	0.884			
Average	0.902	0.914	0.881	0.839
Standard Deviation	0.0467	0.0535	0.0552	0.0697
%Deviation from Overall Mean	2.0%	3.4%	-0.3%	-5.1%
%RSD	5.2%	5.9%	6.3%	8.3%

C₂ SPE & Lightning C₈ HPLC

Analyte	OPC-P			
Lot Number	1	2	3	4
Replicate				
1	0.512	0.5463	0.542	0.628
2	0.576	0.577	0.543	0.507
3	0.596	0.536	0.542	0.561
Overall Mean	0.555			
Average	0.561	0.553	0.542	0.565
Standard Deviation	0.0422	0.0212	0.0007	0.0605
%Deviation from Overall Mean	1.1%	-0.4%	-2.4%	1.8%
%RSD	7.9%	3.8%	0.1%	10.7%

Figure 1. TIC for Phenyl/Hexyl HPLC Column and C2 Extracts

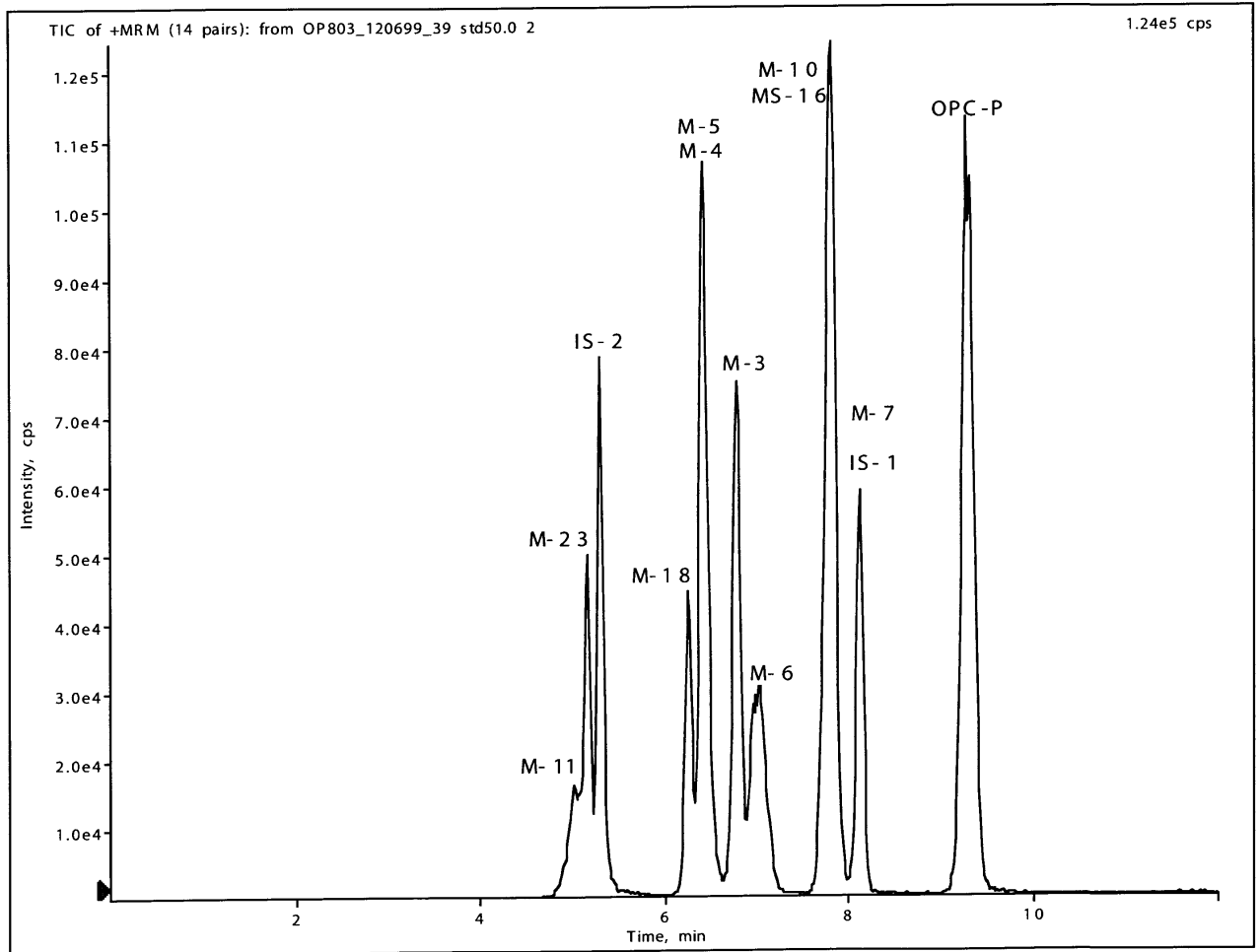


Figure 2. TIC for Asahipak ODP HPLC Column and C2 Extracts

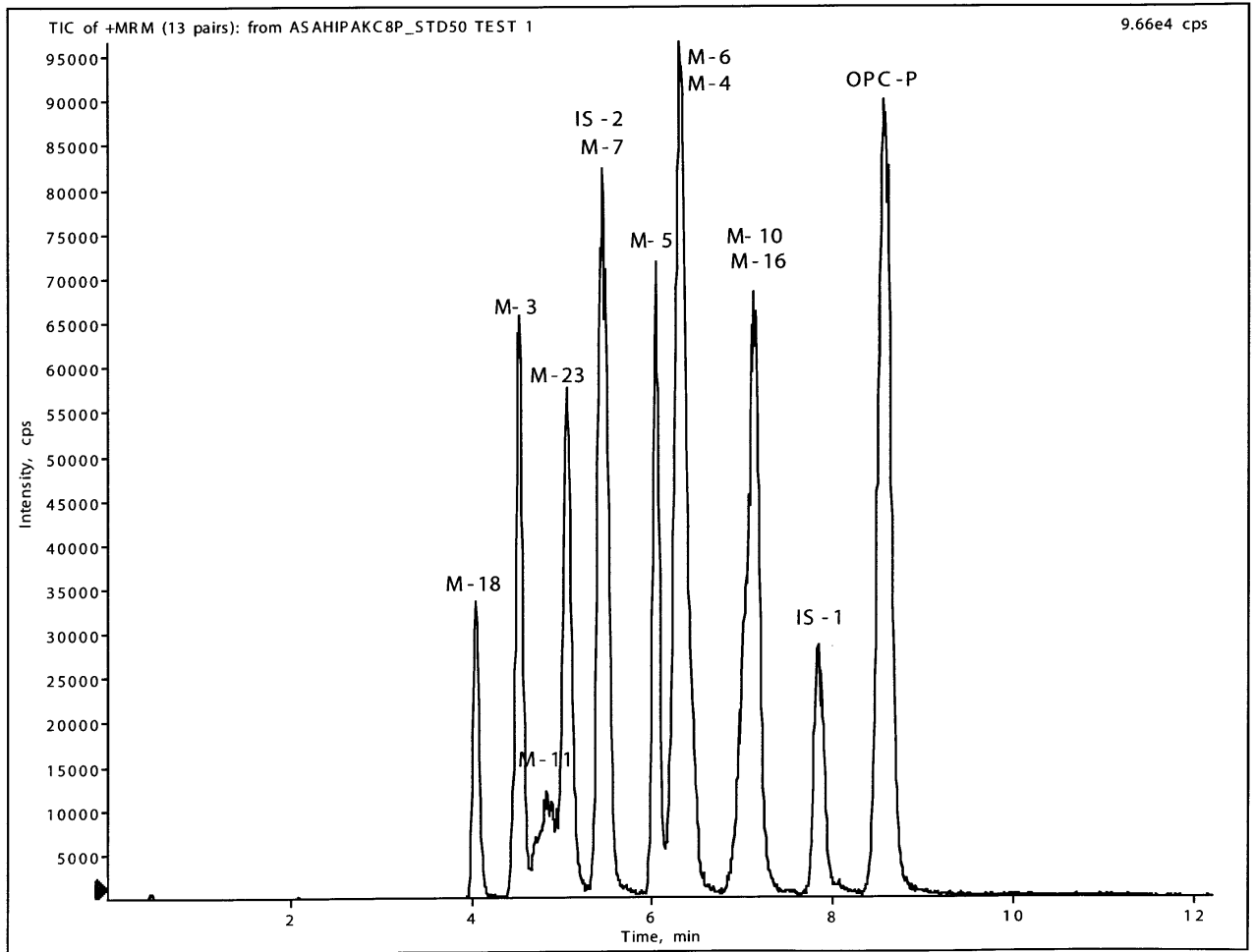


Figure 3. TIC for Lightning C8 HPLC Column and C2 Extracts

