

# Reducing Evaporative Crosstalk in Urine Assays Using 96-well Plates for LC-MS/MS Analysis

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## Background/Introduction

LC-MS/MS analysis of volatile analytes is problematic when 96-well plates are used. Solid phase extraction of urine with high drug concentrations can cause contamination of the elution solvent during evaporation as the compounds volatilize then redistribute in adjacent wells. This phenomenon, known as “evaporative crosstalk”, can generate false positive results. Controlling crosstalk in samples with very high concentrations is particularly difficult for analytes like sympathomimetic amines. Addition of HCl in methanol (MeOH) to extracted samples before evaporation is used to reduce crosstalk. The type of evaporator, conditions and solvents used can all contribute to crosstalk. The Biotage® ACT Adapter (Figure 1, Biotage LLC, Charlotte, NC) was designed to reduce crosstalk during evaporation.

We conducted multiple experiments in our Charlotte applications lab to determine how to control evaporative crosstalk in these high concentration samples using the ACT Plate Adapter and HCl in MeOH.



**Figure 1.** Biotage® ACT Plate Adapter, P/N 414355SP and 2 mL collection plate, P/N 121-5203

## Methods and Materials

Standards were purchased from Cerilliant (Round Rock, TX). Solvents and other reagents were purchased from Reagents (Charlotte, NC). Urine was collected from healthy volunteers. Samples were extracted using 400 µL Biotage® ISOLUTE® SLE+ 96 well plates (Figure 2), and eluent was collected in Biotage® 2 mL square collection plates and evaporated using a Biotage® SPE Dry (Figure 3) at 40 °C upper, 60 °C lower, and gas flows 40 upper and 30 lower. Samples were analyzed using a Shimadzu Nexera UPLC and a Sciex 5500 triple quadrupole mass spectrometer. Eight drugs and metabolites were evaluated: amphetamine, methamphetamine, MDA, MDMA, MDEA, benzoyllecgonine, morphine and hydromorphone.



**Figure 2.** ISOLUTE® SLE+ 400 µL Supported Liquid Extraction Plate, P/N 820-0400-P01

An extraction blank and urine calibrators populated the first column of each plate. Three spiked urine specimens at concentrations between 50,000 and 100,000 ng/mL were placed in different areas of the plate. The rest of the plate was populated with samples of drug free urine. Experiments were done with and without the ACT Plate Adapter, and with different concentrations of HCl in MeOH.

Each extracted sample was 150 µL of urine treated with 165 µL of a master mix designed to be consistent with reagents added for enzyme hydrolysis: pH7 phosphate buffer, methanol (to mimic addition of internal standard solution) and water.

## Methods and Materials (continued)

Samples were not hydrolyzed. Each sample was pretreated with 300 µL of 0.1% ammonium hydroxide. Next, 400 µL of each treated sample were loaded onto individual wells of the 400 µL ISOLUTE® SLE+ plate and extracted using the standard SLE+ protocol. Samples were eluted with 2 x 600 µL of 90:10 dichloromethane:2-propanol. Elution solvent was evaporated using a SPE Dry and reconstituted and analyzed following the LC-MS/MS method in the Biotage® Urine White Paper (Biotage® P/N PPS443, available upon request).



**Figure 3.** Biotage® SPE Dry 96 Plate Evaporator, P/N SD-9600-DHS-NA with ACT Plate Adapter

## Results

Initial experiments showed no evaporative crosstalk for benzoyllecgonine, morphine or hydromorphone with or without the ACT Plate Adapter and the addition of 10 µL of 1 mM HCl in methanol. Some crosstalk was detected in drug free urine samples adjacent to a spiked sample for MDA, MDEA and MDMA but was reduced to <5 ng/mL with the ACT Plate Adapter. Evaporative crosstalk with concentrations in drug free wells between 2 and 200 ng/mL were observed for methamphetamine and amphetamine without the ACT Plate Adapter (Figure 4). This was reduced to 1 to 100 ng/mL when the ACT Plate Adapter was used (Figure 5). This was still too high for many clinical and forensic assays. Further work focused on reducing crosstalk for methamphetamine and amphetamine only.

Next, the concentration of HCl in MeOH was increased. Extractions were performed and either 10 µL of 0.25% HCl in MeOH or 10 µL of 0.5% HCl in MeOH (Figures 6 and 7) were added to separate extracted plates prior to evaporation. Some crosstalk was still observed at both concentrations, but this was reduced to <30 ng/mL for both analytes with 10 µL of 0.5% HCl in MeOH and the ACT Plate Adapter.

## Conclusions

Reducing evaporative crosstalk in extracted urine samples with very high concentrations of volatile analytes is challenging. The ACT Plate Adapter reduced crosstalk for MDA, MDEA and MDMA, methamphetamine and amphetamine, but the correct concentration of HCl in MeOH is required to “salt out” the methamphetamine and amphetamine present in very high concentration samples. The SAMHSA confirmation cutoff for methamphetamine and amphetamine in urine is 250 ng/mL. The combination of 10 µL of 0.5% HCl in MeOH and the use of the ACT Plate Adapter reduced crosstalk to <30 ng/mL. This should be acceptable for most clinical and SAMHSA drug assays using urine specimen volumes of 150 µL or less.

## Plate Maps Showing Evaporative Crosstalk Under Different Conditions

Values are concentrations in ng/mL. Yellow are spiked samples. Pink are concentrations >20 ng/mL in negative samples. Figures 4 and 5 only had methamphetamine and amphetamine spiked in wells C4 and F7.

amphetamine												
	1	2	3	4	5	6	7	8	9	10	11	12
A	blank									0		
B	1									0		
C	5	36	110	100K		3				100K		
D	10	47	10		2	3						
E	50	6	4	7	12	16	10	4		1		
F	100	51	32	31	53	194	50K	5	5	1		
G	500	31	58	28	28	65	84	6				
H	1000	7	5	4	4	7	4	3			1	2

methamphetamine												
	1	2	3	4	5	6	7	8	9	10	11	12
A	blank	1	1	2	1	1	1	1				
B	1	3	5	5	0	2	1					
C	5	23	112	100K		1	2	1		100K		
D	10	51	10	2	2	4	2	1	1			
E	50	7	3	7	9	16	11	3	2			
F	100	53	33	28	57	196	50K	7	3			
G	500	35	56	18	29	70	84	5	2			
H	1000	5	4	2	4	5	6	2	1			

**Figure 4.** Crosstalk without the ACT Plate Adapter and 10 µL of 1 mM HCl in methanol

amphetamine												
	1	2	3	4	5	6	7	8	9	10	11	12
A	blank	6										
B	1	21										
C	5	8	23	100K	64					100K		
D	10		19	66	23							
E	50		14	21		22	38	25	17			
F	100					16	50K	70	12			
G	500					13	48	67	19			
H	1000											

methamphetamine												
	1	2	3	4	5	6	7	8	9	10	11	12
A	blank	2	3	4	5	3	5	2	3	2	2	2
B	1	26	8	8	13	6	4	3	4	4	2	2
C	5	9	19	100K	49	13	8	3	5	100K	2	1
D	10	7	18	41	17	9	11	7	7	6	2	3
E	50	8	13	12	10	16	26	22	12	6	3	1
F	100	3	8	7	7	10	50K	101	12	7	3	0
G	500	3	2	3	6	10	33	57	18	2	3	3
H	1000	3	3	3	4	7	12	15	8	4	1	

**Figure 5.** Crosstalk using the ACT Plate Adapter and 10 µL of 1 mM HCl in methanol

0.25% HCl												
amphetamine												
	1	2	3	4	5	6	7	8	9	10	11	12
A	blank	1	4	1	1	1	1	1	2	1	2	2
B	1	1	2	5	2	2	2	1	1	3	10	8
C	5	1	5	100K	3	2	1	3	8	53K	20	11
D	10	1	2	10	1	3	2	4	5		36	14
E	50	1	2	2	2	5	11	8	6	10	8	6
F	100	2	1	2	2	17	80K	23	10	6	7	4
G	500	3	1	2	3	9	31	21	6	5	8	4
H	1000	2	3	2	2	5	5	10	6	5	2	3

methamphetamine												
	1	2	3	4	5	6	7	8	9	10	11	12
A	blank	2	1	4	2	1	1	1	2	1	2	2
B	1	1	3	8	2	3	1	1	1	3	7	5
C	5	2	7	100K	5	3	2	1	5	53K	18	8
D	10	3	4	31	8	4	2	2	4	1	19	9
E	50	2	4	6	3	6	10	6	4	8	7	6
F	100	1	2	5	3	13	80K	16	5	5	5	2
G	500	1	1	2	2	6	28	15	3	3	6	2
H	1000	1	1	2	3	4	3	6	4	3	2	

**Figure 6.** Crosstalk using the ACT Plate Adapter and 10 µL of 0.25% HCl in methanol

0.5% HCl												
amphetamine												
	1	2	3	4	5	6	7	8	9	10	11	12
A	blank	1	1	3	2	1	1	1	2	3	2	7
B	1	2	3	8	8	4	4	2	6	15	9	5
C	5	2	10	100K	25	7	3	3	9	53K	23	13
D	10	2	8	17	15	8	5	6	8	15	16	8
E	50	1	6	5	10	10	14	8	8	5	7	4
F	100	1	2	5	3	17	80K	11	9	4	1	2
G	500	1	1	3	8	13	29	18	16	10	1	2
H	1000	1	2	1	3	6	12	14	11	8	6	4

methamphetamine												
	1	2	3	4	5	6	7	8	9	10	11	12
A	blank	1	1	2	2	1	1	2	2	2	2	4
B	1	1	3	5	7	2	2	2	6	16	9	7
C	5	2	7	100K	14	4	2	3	9	53K	21	12
D	10	2	5	12	9	4	2	3	6	15	17	6
E	50	1	2	3	5	5	10	5	4	4	5	3
F	100	1	2	2	3	5	80K	9	4	4	2	4
G	500	1	1	1	2	5	13	8	7	4	5	1
H	1000	1	1	1	2	2	5	6	5	4	3	2

**Figure 7.** Crosstalk using the ACT Plate Adapter and 10 µL of 0.5% HCl in methanol

## Other Considerations

Elution volume also plays a role in reducing crosstalk. Reducing the urine volume (and hence sample volume) and using a 200 µL ISOLUTE® SLE+ plate with half the elution volume (2 x 300 µL) reduces the incidence of evaporative crosstalk even further (data not shown).