FIT FOR PURPOSE SAMPLE PREPARATION IN BIOANALYSIS: TOOLS TO REDUCE SOURCES OF MATRIX EFFECTS IN LC/MS/MS ASSAYS

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INTRODUCTION

The need for highly sensitive methods and greater regulatory focus on matrix effects has created the necessity for more sample preparation options for bioanalytical assays (*Table 1*). Depending on method requirements and stage of drug development, different techniques may be appropriate. Protein precipitation (PPT), liquid-liquid extraction (LLE), phospholipid removal (PLR) plates, and reversed-phase (RP) or mixed-mode (MM) solid phase extraction (SPE) are commonly used. The simplest and least expensive technique that meets the assay requirements will be chosen. Examples of methods required for different stages of drug development will be used to highlight the process of determining which sample prep technique is "fit for purpose". The focus will be on novel ways to eliminate sources of matrix effects.

	TECHNIQUES				
ATTRIBUTES	PPT with Filter Plate	PL Removal (PLR) Plate	LLE	Reversed-phase SPE	Mixed-mode SPE
Selectivity	poor	poor	average	average	excellent
Ease of Method Development	excellent	excellent	average	excellent	poor
Generic	excellent	excellent	average	excellent	poor
Reduction of Matrix Effects	poor	average	average	excellent	excellent
General Interference Removal	poor	poor	average	average	excellent
PL Removal	poor	excellent	average	average	excellent
High Sensitivity	poor	average	average	excellent	excellent
Reproducible	excellent	excellent	average	excellent	excellent
Recovery of Diverse Analytes	excellent	excellent	average	excellent	poor
Simplicity of Final Method	excellent	excellent	excellent	average	average
Sample Concentration	poor	poor	poor	excellent	excellent
Matrix	plasma	plasma	plasma, tissue homogenate	all biological matrices	all biological matrices
Cost	excellent	average	average to excellent	average	average

Table 1: Common bioanalytical sample preparation techniques and key attributes

METHODS

Protein precipitation was performed using the Sirocco™ PPT plate in 96-well format with a 3:1 ratio of acetonitrile to plasma. Samples were extracted with a 3:1 ratio of 1% formic acid in acetonitrile to plasma using the Ostro™ PLR plate. LLE extraction was performed using a 5:1 ratio of 100% MTBE or 5% ammonium hydroxide in MTBE to plasma. Reversed-phase SPE was performed using Oasis® HLB and the generic protocol provided by the manufacturer: condition with 200µL of methanol, equilibrate with 200µL of water, load sample, wash with 200µL of 5% methanol in water, and elute in 2x25µL of 100% methanol. In these examples, when mixed-mode SPE was chosen, Oasis® WCX performed the best. The generic method provided by the manufacturer was used in the example containing the small molecules, tamsulosin and doxazosin. The Waters® PST therapeutic peptide protocol was implemented for the final peptide example.

RESULTS/DISCUSSION

One of the major sources of matrix effects is endogenous phospholipids (PLs). Although LLE samples are perceived as clean, this technique does little to remove endogenous PLs. To visually demonstrate remaining PLs, the MRM for 184-> 184 is shown for both traditional LLE and extraction in-well using Ostro PLR plates (*Figure 1*).

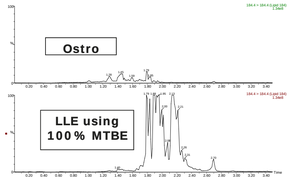

Figure 1: Representative chromatograms of the 184 -> 184 MRM, representing major PLs from Ostro PLR plate and LLE using 100% MTBE

In the first example, a validated method is required for oxycodone and its d6 internal standard for routine analysis of patient samples (*Figure 2*). The desired LLOQ is 50 pg/mL. The method needs to work in both plasma and urine and must clean-up and quantify related compounds and metabolites as well as the primary analyte. To demonstrate the pros and cons from different techniques, recovery and matrix effects for oxycodone and its internal standard were calculated (*Figure 3*). Reversed-phase SPE, Oasis HLB, was chosen because of high analyte recovery, low matrix effects, flexibility to work with urine and plasma matrices, and simplicity of the method. Accuracy and precision requirements were easily met. The assay was linear over four orders of magnitude and the LLOQ of 50 pg/mL was achieved (*Table 2*).

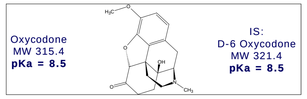

Figure 2: Chemical structure, molecular weight, and pKa of oxycodone and it's d6 internal standard.

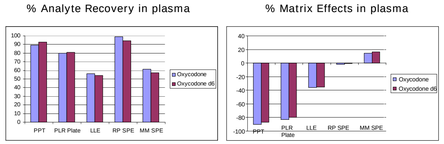

Figure 3: Sample preparation technique screening for oxycodone and it's d6 internal standard.

	Standard conc. ng/mL	Oxycodone Area	IS Area	Response	Calc. conc. ng/mL	%Dev
Standard	0.05	154.3	20459.4	0.008	0.047	-5
Standard	0.1	271.3	17595.4	0.015	0.110	9.6
Standard	0.5	1131.9	17532.5	0.065	0.497	-0.6
Standard	1	2440.3	18100.7	0.135	1.051	5.1
Standard	5	12431.3	18628.3	0.667	5.249	5
Standard	10	24624.5	19441.6	1.267	9.973	-0.3
Standard	50	115082.9	17982.5	6.400	50.441	0.9
Standard	100	214974.1	18094.1	11.881	93.653	-6.3
Standard	500	828731.8	14250.0	58.157	458.476	-8.3
QC	0.25	594.1	18144.6	0.033	0.246	-1.5
QC	0.75	1714.8	17840.9	0.096	0.746	-0.6
QC	7.5	17403.2	17705.8	0.983	7.737	3.2
QC	75	159931.8	16837.3	9.499	74.872	-0.2
QC	0.25	587.429	18774.037	0.031	0.245	-2
QC	0.75	1727.213	18001.096	0.096	0.777	3.6
QC	7.5	17599.27	18394.02	0.957	7.859	4.8
QC	75	159529.25	17495.1	9.119	75	0
QC	0.25	719.038	20666.336	0.035	0.266	6.3
QC	0.75	1914.771	19666.842	0.097	0.757	0.9
QC	7.5	18367.924	19519.512	0.941	7.373	-1.7
QC	75	168356.422	18418.785	9.14	71.683	-4.4

Table 2: Accuracy and precision results for oxycodone calibration curves from 0.05—500 ng/mL in plasma.

In a second example, 26 analogous compounds were screened (see *Figure 4* for representative structures and *Figure 5* for chromatographic separation). As is typical of a screening assay, detection limits were not challenging and a simple generic method was most important. High throughput, direct injection, and removal of phospholipids to maximize instrument uptime were desired. Using a simple, in-well protein crash on the PLR plate, average recovery for the 30 compounds was 84% (*Figure 6*) and the average r^2 value was greater than 0.965. Detection limits were easily met directly injecting the pass-through fraction.

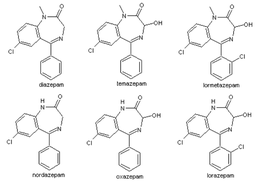

Figure 4: Representative structures of 6 of the 26 structural analogs utilized in this example.

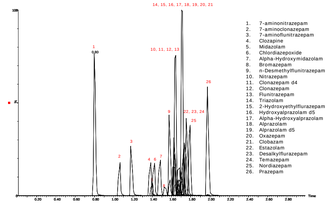

Figure 5: Chromatographic results for 26 structural analogs.

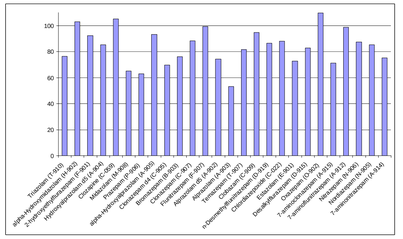

Figure 6: Analyte recovery for 26 structural analogs in plasma, average recovery 84%

In another example, the recovery and matrix effects of tamsulosin and its internal standard, doxazosin (*Figure 7*), are compared for five different sample prep techniques (*Figure 8*). Depending on assay requirements, any of the techniques might be chosen. If the assay requires the simplest sample prep option with no time for method development and reasonable limits of detection, PPT would be the best option. If the assay requires low limits of quantitation, (~5 pg/mL) and high selectivity, mixed-mode SPE is the best option (Figure 9).

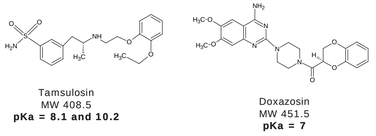

Figure 7: Structures, molecular weights, and pKa's of tamsulosin and its internal standard, doxazosin

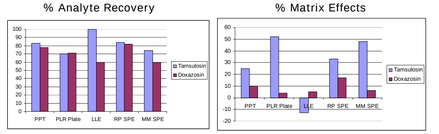

Figure 8: Sample preparation technique screening for tamsulosin and doxazosin in plasma

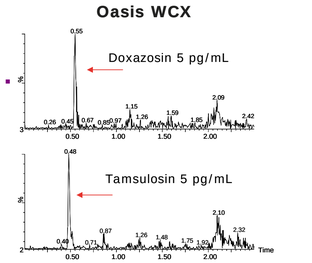

Figure 9: Chromatograms of tamsulosin and doxazosin at 5 pg/mL in plasma facilitated by concentration and direct injection from the Oasis WCX µElution plate

In the final example, a very high sensitivity method must be developed for a peptide drug (*Figure 10*); mixed-mode SPE in a µElution format provided both the required selectivity and the ability to concentrate the sample without evaporation. A detection limit of 1 pg/mL was easily achieved (*Figure 11*). Linear calibration curves were achieved over the range of 1 pg/mL to 20 ng/mL with r^2 values of 0.999 (*Table 3*).

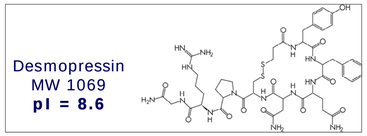

Figure 10: Chemical structure, molecular weight, and pKa of desmopressin.

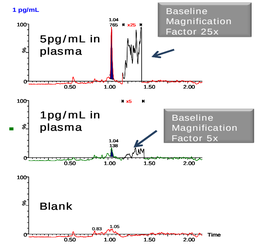

Figure 11: Representative chromatogram of the LLOQ of desmopressin in plasma. The baseline was magnified to demonstrate signal relative to baseline noise in a blank extracted plasma sample

Sample Name	Std. Conc	Area	IS Area	Calc. Conc.	%Dev
Blank human plasma		2.024	20334	0.0003	
0.001 ng/mL	0.001	5.015	17062	0.0010	2.7
0.002 ng/mL	0.002	9.138	17886	0.0018	-9
0.005 ng/mL	0.005	22.187	16283	0.0049	-1.4
0.01 ng/mL	0.01	45.187	17035	0.0096	-3.6
0.02 ng/mL	0.02	113.447	17912	0.0231	15.4
0.05 ng/mL	0.05	240.559	18804	0.0467	-6.6
0.1 ng/mL	0.1	490.062	18654	0.0959	-4.1
1 ng/mL	1	4365.578	15747	1.0125	1.3
5 ng/mL	5	30420.492	20869	5.3239	6.5
10 ng/mL	10	48969.102	17701	10.1042	1
20 ng/mL	20	104231.141	19458	19.5643	-2.2

Table 3: Standard curve statistics from 0.001 to 20 ng/mL for desmopressin in plasma.

CONCLUSIONS

- **Choosing a sample preparation method depends on the final bioanalytical assay requirements**
- **The simplest method which meets the assay needs was chosen**
- **Mixed-mode SPE facilitated routine achievement of low pg/mL LLOQ's for both large and small molecules**

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