



Analysis of Polar Compounds in Biological Matrix with LC/MS/MS via “Normal Phase” LLE for Sample Preparation

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INTRODUCTION

Three sample preparation techniques are commonly used to extract analytes out of biological matrices: protein precipitation (PPT), supported liquid extraction (SLE) or liquid-liquid extraction (LLE), and solid phase extraction (SPE). Among of three, PPT is the most used sample preparation approach in early drug discovery due to less method development time required, therefore fast data turn around time. However, choosing which extraction approach is best is compound dependent in real practice. Analysis of highly hydrophilic molecules in biological matrices presents challenges due to low extraction recovery from biological matrices. Commonly, various solid phase extraction (SPE) procedures are used due to the abundance of different retention mechanisms available to retain the desired analytes, and extract them out of bio-matrices. Often this involves time consuming method development, not suitable for quick turn around. SLE or LLE performed with common organic solvents such as methyl *t*-butyl ether (MTBE), hexanes/ethyl acetate, and 1-chlorobutane commonly cannot apply due to their low LogP index. Also, PPT contributes to the sample extracts a fair amount of phospholipids and dose vehicle that concentrate during the dry-down and reconstitution step. The competition between aqueous and organic phases may result in a lower compound recovery. Also, lower mass compounds can be lost to evaporation during the dry-down period. Herein, we evaluate common approaches and problems in dealing with small hydrophilic molecules in bio-matrices in early stage drug discovery.

METHODS

SAMPLE PREPARATION

Seven small molecules, including both acids and bases with molecular weights less than or close to 200 amu were randomly selected in this study. Selected test compounds were spiked into both blank rat plasma and pre-conditioned 2% polyethylene glycol (PEG) treated rat plasma. The test compounds were extracted from plasma via three sample extraction procedures: 1) Protein precipitation with acetonitrile, followed by either aqueous dilution or dry-down and reconstitution. 2) Liquid/liquid extraction with MTBE, analyzing both aqueous and organic portions. The aqueous layers were centrifuged and directly injected into LC/MS. The organic layer was dried down and reconstituted. 3) Acidic protein precipitation, followed by dilution with buffer. Selected acid solutions were 5% formic acid (FA), 10% acetic acid and 0.1 N HCl. In PPT and LLE approaches, the plasma samples were treated with three different pH conditions: Acidic (5% FA), Neutral (water), and Basic (5% NH₄OH). The purpose is to adjust plasma pH to yield best sample recovery.

LC CONDITIONS

The compounds were divided into two groups, positive ionization mode and negative ionization mode. Several LC conditions were tested on different columns and mobile phases. The final LC separations were achieved on a Phenomenex Polar RP, 2.1 x 50 mm. For the compound group with positive ionization, LC mobile phases were 10 mM ammonium acetate in water (Mobile phase A) and 10mM ammonium acetate in 50:50 ACN:MeOH (Mobile phase B); For the compound group with negative ionization, LC mobile phases were water (Mobile phase A) and 50:50 ACN/MeOH (Mobile phase B). The chosen LC gradient was: hold at 0% A for 0.3 min, from 0 to 100% B in 1.5 min, 0.5 min at 100% B then equilibrate to initial condition for 0.5 min.

MS Conditions and MRM Transitions for Selected Test Compounds

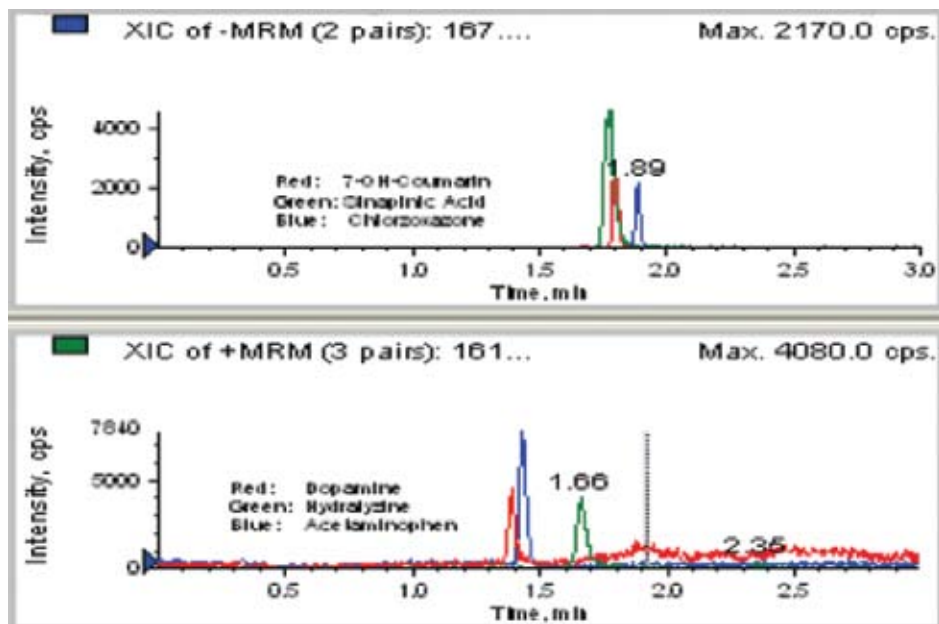
The samples were analyzed by Sciex API-4000 platform coupled with two Shimadzu pumps and a LEAP Autosampler. All compounds were analyzed under electrospray ionization

Name	pKa*	Mass Transition	Ionization Mode
Benzoic acid	4.2	121 → 77	NEG
Sinapinic Acid	6.2	223 → 164	NEG
7-OH-Coumarin	7.8	161 → 132	NEG
Dopamine	10.6	154 → 137	POS
Hydralazine	7.3	161 → 89	POS
Acetaminophen	9.51	152 → 110	POS
Chlorzoxazone	-	168 → 131	NEG



METHODS continued

FIGURE 1: Chromatograms of selected tTest compounds



RESULTS AND DISCUSSION

Evaluation of Method Sensitivity with Different Sample Prep Approaches

In PK bioanalytical sample analysis, we constantly experience low sensitivity analysis of small polar compounds in biological matrices via PPT sample treatment. The polar molecules by their nature, prefer to stay in aqueous phases more than in organic. Because of this, we investigated their extraction by LLE. Instead of collecting of organic supernatant, we focused on the aqueous layer, which may hold more polar analytes in solution. The work was performed with a general LLE approach. The results were compared to PPT.

Sample Extraction	PPT	Dopamine Peak response				Acetaminophen Peak response				
		LLE (MTBE) Org:Aq 2:1		LLE (MTBE) Org:Aq 5:1		LLE (MTBE) Org:Aq 2:1		LLE (MTBE) Org:Aq 5:1		
		Org layer	Aq Layer	Org layer	Aq Layer	PPT	Org layer	Aq Layer	Org layer	Aq Layer
None	32527	No Peak	112466	No Peak	45057	240587	183688	638429	284942	232961
5% FA treated	32230	No Peak	260564	No Peak	85878	233117	360294	958841	204518	267962
5% NH4OH treated	34710	No Peak	34691	No Peak	30769	251002	233797	837776	225958	494922

RESULTS AND DISCUSSION *continued*

Dopamine is a highly basic, polar compound. PPT works well on getting analyte out of the plasma, but method sensitivity is much higher for LLE/aqueous layer injection than signal from the PPT extraction. The low sensitivity from PPT is due to the dilution factor associated with the protein crash and dilution to match initial injection conditions. The dry-down procedure can not be applied here because of severe loss of analyte. There are no peaks observed in the organic layer (MTBE) from LLE. Likely, with the polar nature of dopamine it prefers staying in the aqueous layer. Among three plasma treatment conditions, the samples in aqueous layer with acid treatment show the largest signal intensity in ratio of organic:aqueous 2:1.

Acetaminophen behaves similarly to dopamine in certain ways. The signal in aqueous layer shows the highest intensity but a fairly amount of analyte appears in the organic layer as well.

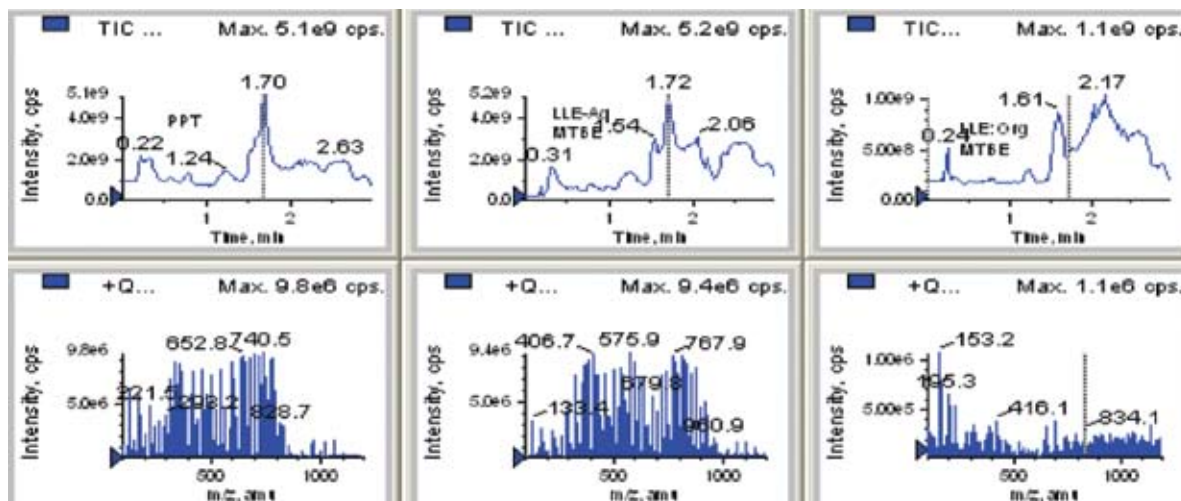
This result indicates that the dopamine can be analyzed using LLE/aqueous injection to boost sensitivity.

Sample Extraction		Sinapinic Acid Peak response				7-OH-Coumarin Peak response				
		LLE (MTBE) Org:Aq 2:1		LLE (MTBE) Org:Aq 5:1		PPT	LLE (MTBE) Org:Aq 2:1		LLE (MTBE) Org:Aq 5:1	
		Org layer	Aq Layer	Org layer	Aq Layer		Org layer	Aq Layer	Org layer	Aq Layer
Plasma Treatment	PPT									
None	935863	123617	10873757	81117	8180055	11187	58112	6350	27086	4021
5% FA treated	1065223	25685613	675272	6911610	247035	20983	35382	1728	12686	711
5% NH4OH treated	594938	187408	2545395	21525	1682002	13300	4594	28304	5892	21992

Results for sinapinic acid indicate that the LLE/aqueous injection yields better sensitivity compared to PPT extraction. There is no significant difference of signal intensities for 7-OH coumarin by PPT or LLE/aqueous injection. For both compounds, the amount of analyte staying in aqueous or organic layers highly depends on the sample pH.

Dose Vehicle Cleaning

Dose vehicle in drug formulation usually causes severe matrix effects resulting in lower sensitivity in sample analysis. In this study, we selected polyethylene glycol, a common dose vehicle in drug formulation, as an example. The test compounds were spiked into the rat plasma with 2% PEG and followed the same sample extraction procedures. For the LC gradient we tested, the PEG elutes from the column almost throughout the entire gradient, with low mass PEG eluting out around 0.4 min and higher mass PEG eluting out in the range from 1.2 to 2.5 minutes. The chromatograms shown below were from negative mobile phases, with positive ionization. The compounds retained at the same retention time are severely affected by PEG. Most of PEG stays in the aqueous layer in LLE process. People should be aware that analysis of the aqueous layer in LLE may not have any advantage in removing dose vehicles from biological matrices.

RESULTS AND DISCUSSION *continued*


Plasma Treatment		None			5% FA Treated			5% NH ₄ OH Treated		
Compound ID	Sample extraction	PEG	no PEG	% RE +PEG/-PEG	PEG	no PEG	% RE +PEG/-PEG	PEG	no PEG	% RE +PEG/-PEG
Acetaminophen	PPT-dil	74578	240587	31.0	75603	233117	32.4	78603	251002	31.3
	PPT-dry	53470	188778	28.3	46168	180643	25.6	53305	190788	27.9
Benzoic Acid	PPT-dil	72757	125777	57.8	126048	132644	95.0	76207	125834	60.6
	PPT-dry	83586	120298	69.5	111272	109077	102.0	90362	120913	74.7
Sinapinic Acid	PPT-dil	152287	935863	16.3	341202	1065223	32.0	115635	594938	19.4
	PPT-dry	189457	704812	26.9	258817	703147	36.8	149162	704345	21.2
7OH-Coumarin	PPT-dil	6238	11187	55.8	7212	20983	34.4	5882	13300	44.2
	PPT-dry	3715	10067	36.9	5932	15878	37.4	5187	8520	60.9

Phospholipid removal

Phospholipids are major components that stay in the extracts and elute late in the columns. It may not affect polar compounds as much as they do for late eluting analytes. However, the lipids require high organic composition in the LC gradient to clean them out of the LC column, otherwise it will accumulate inside the column and cause poor peak shape or retention shifts. PPT approach does not remove phospholipids from extracted samples. With LLE, the majority of phospholipids stay in the aqueous layer when using MTBE as an extraction solvent regardless if the plasma pH is adjusted or not. By switching to a more polar organic extraction solvent, such as ethyl acetate, more lipids will participate into the organic layer. In our study, we tested 1:4 MTBE:Ethyl Acetate as an extraction solvent. The phospholipids response increases ~ 10 fold in the organic layer compared to using only MTBE. This suggests to use ethyl acetate as an extraction solvent in the application of analyzing the aqueous layer in LLE.

RESULTS AND DISCUSSION *continued*

Test Compound	Chlorzoxazone					
Plasma terated with	5 %PEG in plasma			No PEG added		
	None	2.5% FA	2.5% NH4OH	None	2.5% FA	2.5% NH4OH
PPT-dilute injected	18505	19837	18152	15555	16943	14622
PPT-dry-recon-inject	14307	15252	14040	11748	10495	10697
Precentage lost	22.7	23.1	22.7	24.5	38.1	26.8
Test Compound	Acetaminophen					
PPT-dilute injected	74578	75603	78603	240587	233117	251002
PPT-dry-recon-inject	53470	46168	53305	188778	180643	190788
Percentage lost	28.3	38.9	32.2	21.5	22.5	24.0
Test Compound	Dopamine			Hydralyzine		
PPT-dilute injected	32527	32230	34710	131827	648847	57957
PPT-dry-recon-inject	9933	7498	9482	19568	43982	19930
Percentage lost	69.5	76.7	72.7	85.2	93.2	65.6
Test Compound	7-OH-Coumarin					
PPT-dilute injected	6238	7212	5882	11187	20983	13300
PPT-dry-recon-inject	3715	5932	5187	10067	15878	8520
Percentage lost	40.4	17.7	11.8	10.0	24.3	35.9
Test Compound	Benzoic Acid					
PPT-dilute injected	72757	126048	76207	125777	132644	125834
PPT-dry-recon-inject	83586	111272	90362	120298	109077	120913
Percentage lost	-14.9	11.7	-18.6	4.4	17.8	3.9



CONCLUSION

Utilizing LLE sample preparation approach and directly injection of the aqueous layer increased method sensitivity for polar small molecules in biological matrices. The method works for most small molecules since it does not apply a dilution factor or dry-down step.

PPT sample preparation can extract analytes out of biological matrix but yield low recovery due to the dilution factor associated with the protein precipitation step.

Both sample preparation approaches do not clean matrix background either from phospholipids or dose vehicle. A majority of phospholipids and dose vehicle (PEG in this case) still exist in the LLE/ aqueous layer. Increased polarity of organic extraction solvent, such as combining ethyl acetate with MTBE can remove more phospholipids from aqueous layer but not all of them. If matrix effect is a big concern, then alternative sample preparation methods should be developed.

The selected dose vehicle, PEG, showed severe matrix effects among all test compounds.

Low mass analytes show severe loss during the dry-down step. However, the percentage loss is compound dependent. It shows serious loss for dopamine and hydralazine, and thus we recommend no dry-down in the method development of these two compounds.

Increased sensitivity compared to PPT is compound and sample pH dependent. The matrix effects from dose vehicle and phospholipids were reduced due to the majority of dose vehicle and phospholipids staying in the organic layer. SLE is not a suitable approach due to its difficulty with aqueous extraction. LLE involves intensive labor, but it is difficult to automate. We suggest using deep 96-well plates and after centrifugation, setting up the autosampler needle depth to inject from aqueous layer directly.

Acidic protein precipitation works on acidic analytes better than basic. Like PPT, there is not much difference in the behavior of lipids or dose vehicle. There is no retention of basic compounds on the column due to the charged states in acidic conditions. The compounds also need to be buffered back to avoid a low pH that causes the column to degrade too fast.