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Efficient Extraction of Propranolol, Doxepin, and Loperamide in Plasma Using Oasis PRiME MCX

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APPLICATION BENEFITS

- Faster, simplified sample preparation workflow
- Elimination of at least 98% of phospholipids compared to protein precipitation
- No evaporation or reconstitution necessary

WATERS SOLUTIONS

Oasis™ PRiME MCX µElution Plate

ACQUITY UPLC™ I-Class System (FTN)

ACQUITY UPLC CSH C_{18} 130Å, 1.7 µm, 2.1 x100 mm Column

Xevo™ TQD Mass Spectrometer

MassLynx™ Software

Oasis MCX µElution Plate

KEYWORDS

Oasis PRiME MCX, SPE, Propranolol, Doxepin, Loperamide, Phospholipid removal, LC-MS/MS

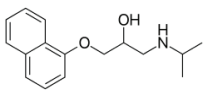
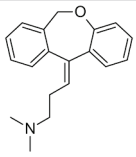
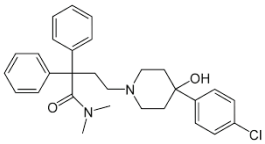
INTRODUCTION

Bioanalysis plays an essential role in drug discovery and development, of which sample preparation is a critical step in order to achieve reliable results.[1] Speed, method robustness and cost are important factors to consider when developing an analytical method for a high-throughput laboratory.

Oasis PRiME MCX, a mixed-mode strong cation-eXchange solid-phase extraction (SPE) Product, has been developed to generate cleaner extracts for basic compounds compared to other sample preparation techniques, by the removal of phospholipids. In this application, faster 3- and 4-step protocols, that eliminate the conditioning and equilibration steps compared to conventional solid-phase extraction (SPE), were used to extract analytes from human plasma in a 96-well plate µElution format.

The structure and properties of propranolol, doxepin, and loperamide are shown in Table 1. The analytes' low molecular weights and pKa in the range 9.41 to 9.76 makes them amenable to reverse phase chromatography and detection by electrospray positive ion mass spectrometry. The high pKa also means these drugs are well suited to sample preparation using cation exchange.

Table 1. Structure and properties of Propranolol, Doxepin, and Loperamide.

Compound	Structure	Formula	Molecular weight	pKa
Propranolol		$C_{16}H_{21}NO_2$	259.35	9.67
Doxepin		$C_{19}H_{21}NO$	279.38	9.76
Loperamide		$C_{29}H_{33}ClN_2O_2$	477.05	9.41

This application note describes the analysis of the three pharmaceutical drugs using four sample extraction protocols; protein precipitation, Oasis MCX and Oasis PRiME MCX (3-step) and Oasis PRiME MCX (4-step) to compare analyte recovery and matrix effects including phospholipid removal.

EXPERIMENTAL

SAMPLE PREPARATION

In-house calibrators were prepared by spiking propranolol, doxepin and loperamide (Sigma Aldrich, Dorset, England) into pooled human plasma (Sera Laboratories, West Sussex, United Kingdom) over the concentration range 1.0–100 ng/mL. Quality Control (QC) samples were also prepared in-house at 7.5, 30, and 75 ng/mL (referred to as QC Low, QC Mid, and QC High respectively).

SAMPLE EXTRACTION

1. Protein precipitation

Plasma calibrator/QC (100 µL) was transferred to a graduated microfuge tube. The proteins were precipitated by the addition of methanol (300 µL). Samples were capped and vortexed for 30 seconds prior to centrifugation for 5 minutes at 13000 rpm.

2. Oasis MCX

Plasma calibrator/QC (100 µL) was transferred to a graduated microfuge tube. The sample was treated by the addition of 4% phosphoric acid (100 µL), to disrupt protein binding, protonate the analytes and enable ion exchange interactions. The samples were capped and vortexed for 30 seconds prior to centrifugation for 5 minutes at 13000 rpm. The supernatant was transferred to a 96-well Oasis MCX µElution Plate that had been conditioned with methanol and equilibrated with water. The plate was washed with 2% formic acid (aqueous) followed by 100% methanol. Samples were eluted with 5% ammonium hydroxide in methanol (2 x 25 µL) followed by water (50 µL). The plate was sealed and lightly vortexed for 30 seconds before analysis.

3. Oasis PRiME MCX (4-step protocol)

Plasma calibrator/QC (100 µL) was transferred to a graduated microfuge tube. The sample was treated by the addition of 4% phosphoric acid (100 µL), to disrupt protein binding, protonate the analytes and enable ion-exchange interactions.

The samples were capped and vortexed for 30 seconds prior to centrifugation for 5 minutes at 13000 rpm. The supernatant was transferred to a 96-well Oasis PRiME MCX µElution Plate and washed with wash 1 (100 mM ammonium formate with 2% Formic acid) and wash 2 (100% Methanol). Samples were eluted with 5% ammonium hydroxide in methanol (2 x 25 µL) followed by water (50 µL). The plate was sealed and lightly vortexed for 30 seconds before analysis.

The solid-phase extraction procedures are summarized in Figure 1.

4. Oasis PRiME MCX (3-step protocol)

Plasma calibrator/QC (100 µL) was transferred to a graduated microfuge tube. The sample was treated by the addition of 200 mM ammonium formate with 4% phosphoric acid (100 µL), to disrupt protein binding, protonate the analytes and enable ion-exchange interactions.

The samples were capped and vortexed for 30 seconds prior to centrifugation for 5 minutes at 13000 rpm. The supernatant was transferred to a 96-well Oasis PRiME MCX µElution Plate and washed with 100% methanol. Samples were eluted with 5% ammonium hydroxide in methanol (2 x 25 µL) followed by water (50 µL). The plate was sealed and lightly vortexed for 30 seconds before analysis.

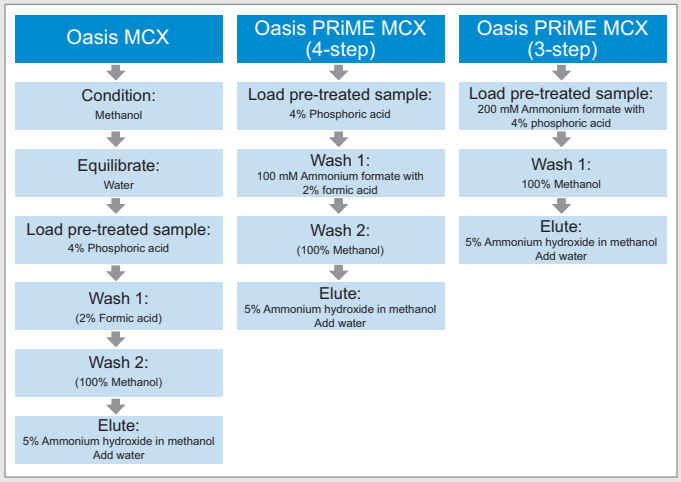


Figure 1. Oasis PRiME MCX comparison with traditional SPE extraction methodology, with no conditioning and equilibration, sample extraction is simplified to just 3- or 4-steps.

LC conditions

LC system:	ACQUITY UPLC I-Class System (FTN)
Column:	ACQUITY UPLC CSH C_{18} 130Å, 1.7 µm, 2.1 x100 mm, (P/N 186005297)
Mobile phase A:	Water with 0.1% formic acid
Mobile phase B:	Methanol
Wash solvent:	80% $Methanol_{(aq)}$ and 0.1% formic acid
Purge solvent:	20% $Methanol_{(aq)}$ and 0.1% formic acid
Seal wash:	20% $Methanol_{(aq)}$
Column temp.:	45 °C
Sample temp.:	10 °C
Injection vol.:	10 µL
Gradient:	See Table 2
Run time:	5.0 minutes (approximately 5.0 minutes injection to injection)

MS conditions

System:	Xevo TQD
Resolution:	MS1 and MS2 (0.75 FWHM)
Acquisition mode:	Multiple Reaction Monitoring (MRM) (see table 3 for details)
Polarity:	ESI positive
Capillary voltage:	1.0 kV
Cone voltage:	See table 3
Source temp.:	150 °C
Desolvation temp.:	600 °C
Desolvation gas flow:	1000 L/Hr
Cone gas flow:	100 L/Hr
Dwell time:	0.02 seconds
Inter-scan delay:	0.02 seconds
Inter-channel delay:	0.01 seconds

Table 2. Gradient table for the chromatographic separation.

Time (min)	Flow rate (mL/min)	A (%)	B (%)	Curve
Initial	0.4	90	10	Initial
3.00	0.4	5	95	6
3.50	0.4	90	10	11

Table 3. Guideline MRM parameters.

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone (V)	Collision (eV)
Propranolol	260.3	116.1	38	18
Doxepin	280.3	107.0	40	24
Loperamide	477.3	266.1	46	26

Data management

MassLynx® v4.1 with XS Application Manager

RESULTS AND DISCUSSION

RECOVERY AND PRECISION

The extraction recovery for protein precipitation, Oasis MCX, Oasis PRiME MCX (4-step), and Oasis PRiME MCX (3-step) sample extraction were determined by the analysis of three QC samples across the concentration range in replicates of 6. The extraction recovery was calculated using the following equation:

$$\text{Recovery (\%)} = \left(\frac{\text{Response}_{\text{PRE-Spiked Extracted Sample}}}{\text{Response}_{\text{POST-Spiked Extracted Sample}}} \right) \times 100$$

Across the three analytes, good extraction recoveries (overall mean % Recovery range 81.2 to 99.1) and precision (% RSD range 3.1 to 9.3) were obtained for Oasis PRiME MCX (3- step), Oasis PRiME MCX (4-step), and Oasis MCX. Good precision was also obtained for protein precipitation (% RSD range 7.8 to 8.6) but the % Recovery was very poor compared to any of the Oasis MCX sample preparation protocols (overall mean % Recovery range 30.7 to 32.1) (Table 4).

Table 4. Precision performance (% RSD) and extraction recovery (% Recovery) for protein precipitation, Oasis MCX, Oasis PRiME MCX (4-step), and Oasis PRiME MCX (3-step) Protocols.

	Protein precipitation		Oasis MCX		Oasis PRiME MCX (4-step)		Oasis PRiME MCX (3-step)	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
QC Low								
Propranolol	28.5	1.3	86.0	3.8	90.9	4.7	75.1	12.1
Doxepin	29.0	1.6	91.1	5.2	97.5	2.3	86.0	7.6
Loperamide	27.9	1.0	97.2	2.4	96.5	3.0	90.0	6.1
QC Mid								
Propranolol	35.0	1.2	94.8	2.5	96.1	2.7	85.4	5.3
Doxepin	34.9	1.0	96.4	3.1	102.8	1.4	94.5	2.1
Loperamide	33.5	0.9	100.4	1.7	102.1	2.0	94.8	2.3
QC High								
Propranolol	32.3	1.0	86.0	3.8	93.9	2.8	83.1	5.6
Doxepin	32.5	0.9	92.9	3.2	97.1	1.8	91.9	2.8
Loperamide	30.6	0.8	96.8	3.6	94.6	2.1	92.0	3.6
QC Overall Mean								
Propranolol	31.9	8.6	91.5	5.5	93.6	4.0	81.2	9.3
Doxepin	32.1	7.9	93.4	4.4	99.1	3.2	90.8	6.0
Loperamide	30.7	7.8	98.1	3.1	97.7	4.0	92.3	4.5

MATRIX EFFECTS AND PHOSPHOLIPIDS REMOVAL

The matrix effects for protein precipitation, Oasis MCX, and Oasis PRiME MCX sample extraction were determined by the analysis of three samples across the concentration range in replicates of 6. The matrix effects were calculated using the following equation:

$$\text{Matrix Effects (\%)} = \left(\left(\frac{\text{Response}_{\text{Post-Spiked Extracted Sample}}}{\text{Response}_{\text{In Absence of Matrix}}}\right) - 1\right) \times 100$$

The peak area in the presence of matrix refers to an extracted matrix sample in which the analyte was added post-extraction. The peak area in the absence of matrix refers to a solvent solution of the analyte.

There was significant matrix effects observed when using protein precipitation; the mean matrix effect was -51.6% (range -47.8% to -54.5%) indicating severe ion suppression. Using mixed-mode strong cation-exchange solid-phase extraction the matrix effects observed were reduced, Oasis MCX mean matrix effect was -17.2% (range -15.3 to -18.4%). The mean matrix effects were further reduced to less than 10% when using Oasis PRiME MCX (4-step) -7.8% (range -5.6% to 11.0%) and Oasis PRiME MCX (3-step) -6.9% (range -3.7% to -10.4%).

To assess phospholipid removal a parent ion scan experiment was conducted monitoring parent ions of *m/z* 184 in plasma sample extracts using the four extraction protocols. The phospholipid peak areas were normalized to the protein precipitation extract, which contained the highest levels. Figure 2 clearly demonstrates up to 98% phospholipid removal when compared to protein precipitation when using Oasis PRiME MCX Protocols. Oasis MCX still performs well removing up to 86% of the phospholipids when compared to protein precipitation.

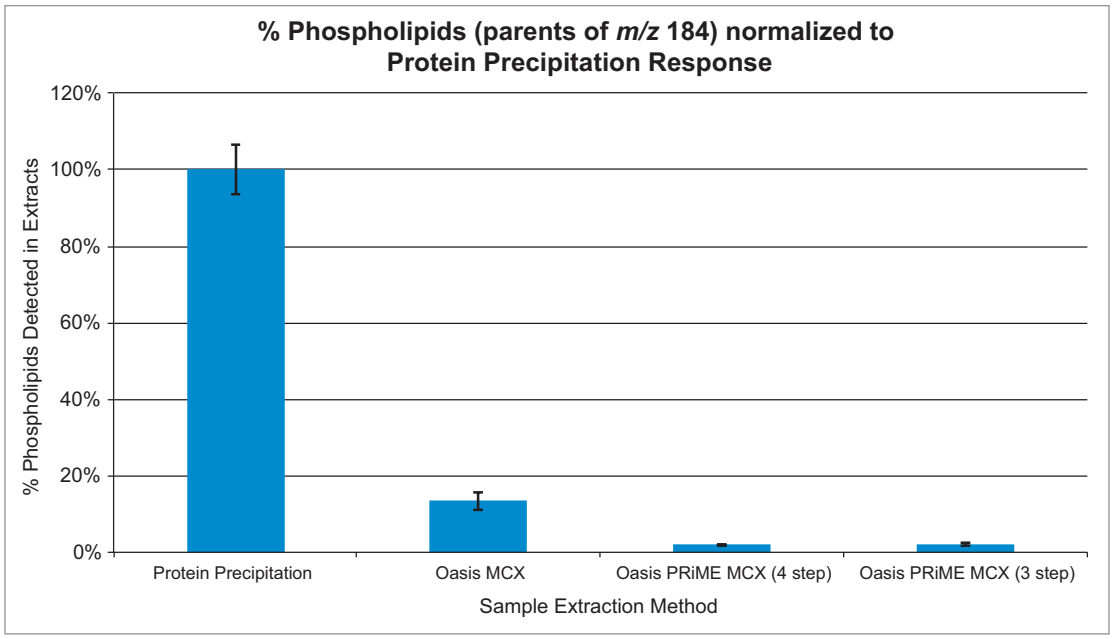


Figure 2. Bar chart to demonstrate the levels of phospholipid present in the extracted plasma sample when using protein precipitation, Oasis MCX, Oasis PRiME MCX (4-step), and Oasis PRiME MCX (3-step).

CONCLUSIONS

Oasis PRiME MCX (3-step) and Oasis PRiME MCX (4-step) sample preparation protocols remove 98% of phospholipids compared to protein precipitation, while, providing reproducible results and cleaner extracts (less than -10% matrix effects). This is an improvement on the Oasis MCX sample extraction protocol which only removes 86.6% of the phospholipids but has comparable precision and % recovery with -17.2% matrix effects.

Even though protein precipitation is an inexpensive form of sample preparation, the matrix effects indicate that inefficiencies (poor recovery and high matrix effects) of the sample clean-up which may lead to poor robustness of the bioanalytical method.

The µElution format with the Oasis PRiME MCX Protocols enable a faster workflow, through the use of a simplified protocol and direct injection of the plasma extract without the need for evaporation or reconstitution. This SPE format and extraction protocol can be easily automated using a liquid handling robot to improve laboratory workflow, eliminate transcription errors, and allow for sample tracking capabilities.

Reference

1. Mohammad MaHDI Moeina, Aziza El Beqqalib, Mohamed Abdel-Rehimb. Bioanalytical method development and validation: Critical concepts and strategies. *J Chromatography B*. 2017; 1043:3–11.

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