Application Note

Forensic Testing

Quantitative Determination of Drugs of Abuse in Human Whole Blood by LC/MS/MS Using Agilent Captiva EMR—Lipid Cleanup

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Abstract

Agilent Captiva Enhanced Matrix Removal—Lipid (EMR—Lipid) is the second generation of EMR—Lipid products, and is implemented in a convenient SPE cartridge or 96-well plate. This study demonstrates the application of Captiva EMR—Lipid 96-well plate for the quantitative determination of 24 representative drugs of abuse in human whole blood by LC/MS/MS. Samples were prepared using in-well protein precipitation (PPT) to remove proteins, followed by Captiva EMR—Lipid cleanup to remove lipids. The protocol was modified by adding whole blood samples first, followed by crashing solvent to encourage thorough in-well PPT. The entire sample treatment was performed as a batch in the 96-well plate, and sample elution was done by either centrifugation or positive pressure manifold. The entire process is simple and quick, preparing 96 samples within two hours. The highly efficient matrix cleanup results in >99 % phospholipid removal, which reduces matrix ion suppression effect and system contamination. The quantitative method was verified by three-day accuracy and precision runs, and delivered exceptional accuracy (100 ±20 %) and precision (RSD <15 %) for all spiking levels, limits of quantitation (LOQ) of 0.1 to 0.5 ng/mL in whole blood, and linear calibration curves with R^2 >0.995. The results demonstrate that the established protocol using in-well PPT followed by Captiva EMR—Lipid cleanup provides significant improvement on the reliable quantitative determination of drug of abuse compounds in human whole blood.

Introduction

In forensic toxicology, the demand for fast and reliable screening and quantitative determination of drugs of abuse (DoA) in biological specimens is steadily increasing[1–3]. This is primarily due to the increasing number of drugs of abuse as well as samples submitted for analysis. Traditionally, urine was the sample of choice for screening and identification. However, the metabolites of these drugs had to be identified, adding more complexity and uncertainty for quantitative testing. The analysis of blood samples including whole blood, plasma, and serum has increased in value. Initially, plasma was the major sample matrix of choice for quantitation, but with the improvement in sample preparation and instrument detection techniques, whole blood has become a viable matrix for identification and quantification. Compared to urine, there are advantages to using blood as a specimen type for analysis.

- First, drugs can be detected just after intake before metabolism and filtration by the body.
- Second, blood is relatively homogeneous since physiological parameters vary within narrow limits.
- Third, blood samples are mandatory in cases of driving under the influence of drugs (DUID) tests several European countries and in some of the United States[4].

Therefore, reliably quantitative determination of DoA in blood matrices, especially in whole blood, is important in regular toxicology analysis.

Toxicological tests for DoA by instrumentation have been done using gas chromatography/mass spectrometry (GC/MS) and liquid chromatography tandem mass spectrometry (LC/MS/MS). However, GC/MS methods usually are time-consuming, and frequently require chemical derivatization of analytes before analysis[5]. The advantage of using LC/MS/MS analytical techniques is the ability for faster testing and higher sensitivity, selectivity, and stability[6]. Sample preparation methods for systematic toxicology analysis include liquid-liquid extraction (LLE), solid-phase extraction (SPE), and supported liquid extraction (SLE). These methods can be labor-intensive, time-consuming, and use of large volumes of toxic solvents.

Agilent Enhanced Matrix Removal—Lipid (EMR—Lipid) sorbent is a novel sorbent material that selectively removes major lipid classes from sample matrix without unwanted analytes loss. The lipid removal mechanism provided by EMR—Lipid sorbent is based on the combination of size exclusion and hydrophobic interaction between lipid compounds and the unique sorbent. This interaction mechanism provides highly selective and efficient removal of phospholipids and other lipids from biological fluids after PPT. The second generation of EMR—Lipid sorbent packed in an SPE cartridge/plate format enables cleanup by simply passing the sample through the sorbent. The phospholipids removal efficacy in biological fluids and the quantitative determination of representative medicinal drugs in human serum were demonstrated using Captiva EMR—Lipid 96-well plate for in-well PPT and subsequent flowthrough cleanup[7,8]. To demonstrate the feasibility of using Captiva EMR—Lipid 96-well plate for forensic tests, this study selected 24 common DoA compounds. Table 1 lists the chemical properties and structures of the selected compounds. The method was verified using three-day accuracy and precision tests as well as matrix cleanup evaluation.

Experimental

Reagent and chemicals

All reagents and solvents were HPLC or analytical grade. Acetonitrile (ACN) was from Honeywell (Muskegon, MI, USA). Reagent grade formic acid (FA) was from Agilent (p/n G2453-85060). Ammonium acetate and ammonium hydroxide were from Sigma-Aldrich (St. Louis, MO, USA). Mixed DoA standard stock solution, 1 µg/mL in MeOH, was from Agilent (p/n 5190-0470-1). Human whole blood was from Biological Specialty Corp., (Colmar, PA, USA). Internal standard (IS) stock solutions, 1 mg/mL in MeOH or ACN, were bought from Cerilliant (Round Rock, TX, USA).

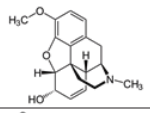
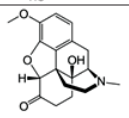
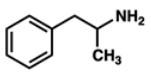
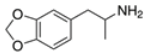
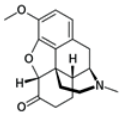
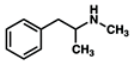
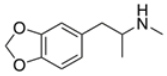
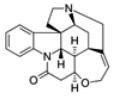
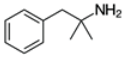
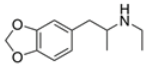
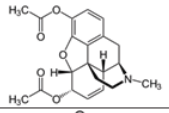
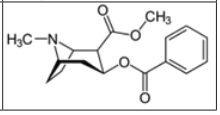
Standards and solutions

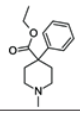
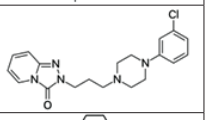
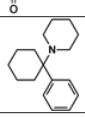
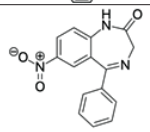
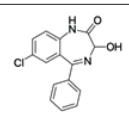
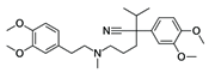
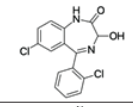
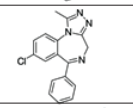
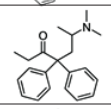
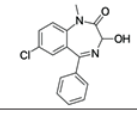
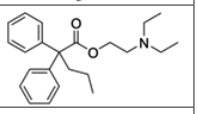
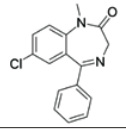
A combined DoA standard stock solution and individual IS stock solutions were used to prepare standard and IS spiking solutions. Standard spiking solution was prepared in 20:80 MeOH/water at 200 ng/mL, and was used to spike calibration standards and QC samples. The IS spiking solution was prepared by diluting individual IS stock solutions with 20:80 MeOH/water at 2 µg/mL, and was used to spike into samples directly.

Mobile phase A, 5 mM ammonium acetate buffer with 0.1 % formic acid (FA), was prepared by dissolving 385.3 mg of ammonium acetate into 1 L of Milli-Q water, then adding 1 mL of FA. Mobile phase B, 0.1 % FA in ACN, was made by adding 1 mL of FA into 1 L of ACN.

A solution of 1 % ammonium hydroxide (NH_4OH) in 95:5 ACN/MeOH was prepared freshly by adding 400 µL of NH_4OH into 40 mL of premixed 95:5 ACN/MeOH. This solution was kept at –20 °C until use.

Table 1. List of DoA compounds, drug class, logP, pKa, and structures.

DoA compound	Class	pKa	LogP	Structure
Codeine	Opiod	8.21	1.19	
Oxycodone	Opiod	8.28	0.3	
Amphetamine	Amphetamine	10.2	1.76	
MDA	Amphetamine	9.67	1.64	
Hydrocodone	Opiod	8.23	1.2	
Methamphetamine	Amphetamine	9.87	2.07	
MDMA	Amphetamine	9.9	2.28	
Stychnine	Alkaloid	8.26	1.93	
Phentermine	Amphetamine	10.14	1.9	
MDEA	Amphetamine	8.52	2.14	
Heroin	Opiod	7.95	1.98	
Cocaine	Alkaloid	8.61	2.3	

DoA compound	Class	pKa	LogP	Structure
Meperidine	Opiod	8.59	2.72	
Trazodone	Triazolopyridine	6.14	2.9	
PCP	Arylcyclohexylamine	8.29	4.69	
Nitrazepam	Benzodiazepine	3.2, 10.8	2.25	
Oxazepam	Benzodiazepine	1.55, 10.9	2.24	
Verapamil	Phenylalkylamine	8.92	3.79	
Lorazepam	Benzodiazepine	13	2.39	
Alprazolam	Benzodiazepine	11.6	2.12	
Methadone	Opiod	8.93	3.93	
Temazepam	Benzodiazepine	10.86	2.19	
Proadifen	–	6.8	5.1	
Diazepam	Benzodiazepine	3.4	2.82	

A 5 mM ammonium acetate solution was made by dissolving 77.06 mg of ammonium acetate into 200 mL of Milli-Q water. This reconstitution solution was prepared by mixing the above buffer and ACN at ratio of 8:2. A 80:20 ACN/water solution was made by mixing 80 mL of ACN with 20 mL of water.

Equipment and material

Equipment used for sample preparation included:

- CentraCL3R centrifuge (Thermo IEC, MA, USA)
- MultiTube vortexer (VWR, PA, USA)
- Eppendorf pipettes and repeater
- SPE Dry 96 evaporator
- Agilent PPM-96 (p/n 5191-4116)
- Agilent Captiva EMR 96-well plate (p/n 5190-1001)
- Agilent Captiva 96-well 1 mL collection plate (p/n A696001000)
- Agilent Captiva 96-well plate cover, 10/pk (p/n A8961007)

Instrument conditions

The samples were run on an Agilent 1290 Infinity LC system consisting of an Agilent 1290 Infinity binary pump (G4220A), an Agilent 1290 Infinity high-performance autosampler (G4226A), and an Agilent 1290 Infinity thermostatted column compartment (G1316C). The LC system was coupled to an Agilent G6490 triple quadrupole LC/MS (G6490A) system equipped with an Agilent Jet Stream iFunnel electrospray ionization source. Agilent MassHunter workstation software was used for data acquisition and analysis. The instrument method details are shown in Table 2.

Table 2. Instrument method conditions.

LC Conditions	
Column	Agilent InfinityLab Poroshell 120, EC-C8, 100 × 2.1 mm, 2.7 µm (p/n 695775-906(T)) Agilent InfinityLab Poroshell 120 guard, EC-C18, 2.1 × 5 mm, 2.7 µm (p/n 821725-911)
Flow rate	0.5 mL/min
Column temperature	60 °C
Injection volume	5 µL
Mobile phase	A) 5 mM ammonium acetate buffer with 0.1 % FA in water B) 0.1 % FA in acetonitrile
Needle wash	1:1:1:1 ACN/MeOH/IPA/H_2O with 0.2 % FA
Gradient	Time (min) %B Flow rate (mL/min) 0 10 0.5 0.5 10 0.5 3.0 50 0.5 4.0 95 0.5 6.0 100 0.5
Stop time	6 minutes
Post time	2 minutes
MS Conditions	
Gas temperature	120 °C
Gas flow	14 L/min
Nebulizer	40 psi
Sheath gas heater	400 °C
Sheath gas flow	12 L/min
Capillary	3,000 V
iFunnel parameters	High-pressure RF: 90 V (POS), 90 V (NEG) Low-pressure RF: 70 V (POS), 60 V (NEG)
Data acquisition	dMRM
Acquisition polarity	Positive

Refer to Table 3 for analyte parameters and Figure 1 for LC/MS/MS chromatogram at the LOQ of DoA in human whole blood.

Calibration standards and QC samples preparation

Calibration curve standards were prepared in whole blood using the standard spiking solution of 200 ng/mL in 20:80 MeOH/water. The dynamic range for the calibration curve was from 0.1/0.5 to 20 ng/mL, including 0.1, 0.5, 1, 5, 10, 15, 20 ng/mL. These standards were prepared by spiking an appropriate volume of standard spiking solution into the whole blood blank, then vortexing well. Three levels of quality control (QC) samples were run for accuracy and precision method verification tests, including lowest limit of quantitation (LLOQ) of 0.1 or 0.5 ng/mL, mid QC of 1 ng/mL or 5 ng/mL, and highest limit of quantitation (HLOQ) of 20 ng/mL. These QC samples were prepared by spiking an appropriate volume of standard spiking solution into the sample whole blood blank. All calibration standards and QCs were prepared in 2-mL snap-cap tubes.

Table 3. List of DoA analytes and IS retention time and MRM conditions.

Analyte	Internal standard used	Retention time (min)	Precursor ion (*m/z*)	Product ion (*m/z*)			
				Quant ion	CE (V)	Qual ion	CE (V)
Codeine	IS 1	1.19	300.2	128.1	60	165.1	40
Oxycodone	IS 1	1.60	316.2	241.1	28	256.1	24
Amphetamine-D_5 (IS 1)		1.68	141.1	93.0	13	124.1	5
Amphetamine	IS 1	1.70	136.1	91.1	20	65.0	40
MDA	IS 1	1.78	180.1	163.1	4	105.1	24
Hydrocodone	IS 1	1.89	300.2	128.1	60	171.1	40
Methamphetamine	IS 1	2.04	150.1	91.1	20	119.1	8
MDMA	IS 1	2.08	194.1	163.1	8	105.1	24
Strychnine	IS 1	2.28	335.2	184.1	40	156.1	40
Phentermine	IS 2	2.32	150.1	91.0	20	65.1	48
MDEA	IS 2	2.42	208.1	163.1	8	105.1	24
Heroin	IS 2	2.93	370.2	328.2	20	165.1	40
Cocaine	IS 2	3.04	304.2	182.1	16	82.0	48
Cocaine-D_3 (IS 2)		3.04	307.2	185.1	30	82.0	48
Meperidine	IS 2	3.10	248.2	220.1	20	174.1	16
Trazodone	IS 2	3.31	372.2	176.1	24	148.1	36
PCP	IS 2	3.43	244.2	86.2	8	91.1	36
Nitrazepam	IS 2	3.97	282.1	180.1	40	236.1	24
Oxazepam	IS 2	4.00	287.1	241.1	20	104.1	40
Verapamil	IS 2	4.01	455.3	165.1	28	150.1	48
Lorazepam	IS 2	4.08	321.0	229.1	32	275.0	20
Alprazolam	IS 2	4.11	309.1	205.1	48	281.1	40
Methadone	IS 2	4.11	310.2	265.2	12	105.0	28
Temazepam	IS 2	4.31	301.1	177.0	44	255.1	16
Proadifen	IS 2	4.48	354.2	167.1	40	91.1	40
Diazepam	IS 3	4.55	285.1	193.1	32	154.1	24
Diazepam-D_5 (IS 3)		4.54	290.1	198.1	32	154.1	24

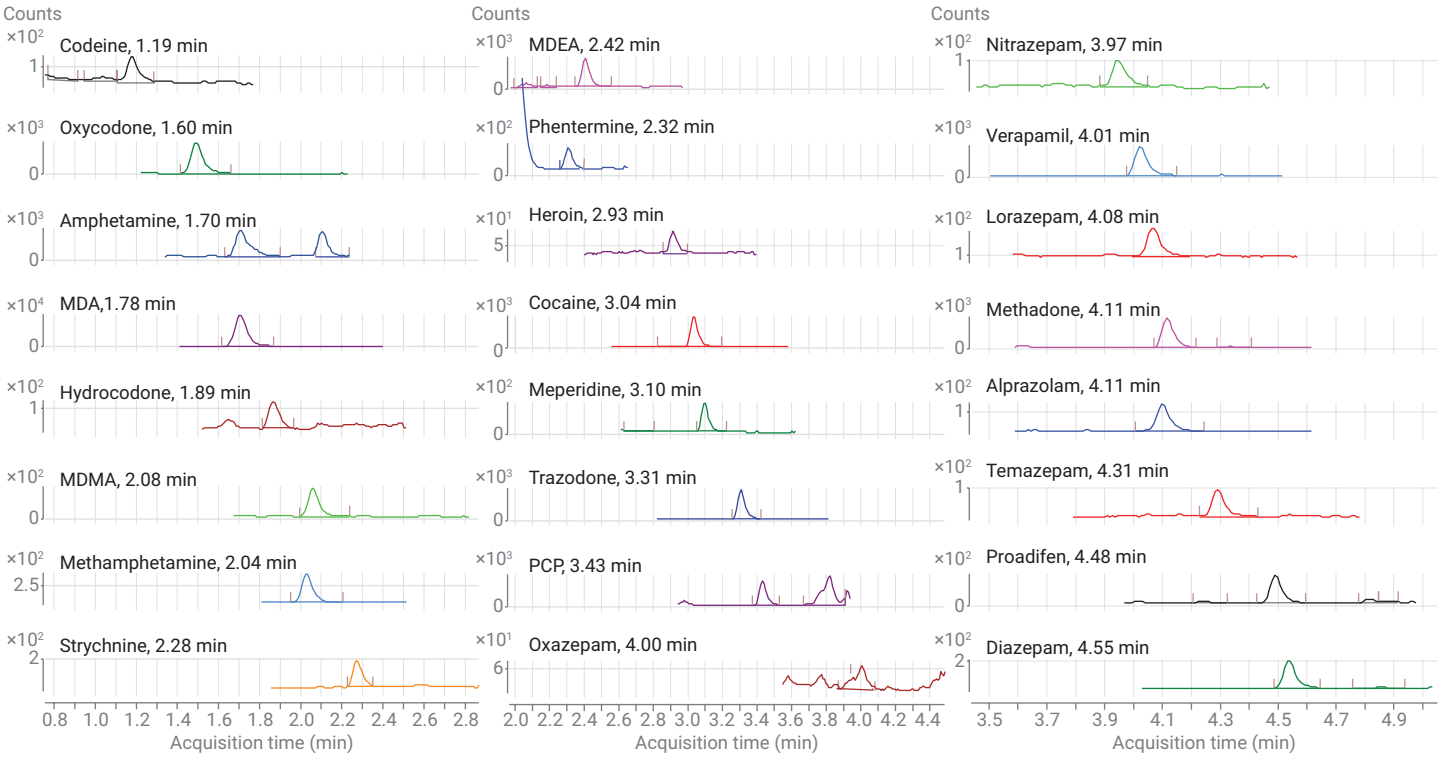


Figure 1. LC/MS/MS chromatogram (dMRM) for human whole blood samples fortified at the LOQ level of DoA in human whole blood (0.1 ng/mL except amphetamine, heroin, and lorazepam with 0.5 ng/mL). Samples were extracted by in-well protein precipitation, followed by Captiva EMR—Lipid cleanup.

Method verification

Method verification was performed through three-day accuracy and precision (A&P) runs. Calibration standards and QCs were prespiked appropriately. Samples were aliquoted into an EMR—Lipid plate in the following sequence:

1. Double matrix blank
2. Matrix blank (spiked with IS)
3. First set of calibration standards
4. 2–3 Matrix blanks
5. LOQs (n = 6)
6. Mid QCs (n = 6)
7. HLOQ (n = 6)
8. 2 to 3 Carryover matrix blanks
9. Double matrix blank
10. Matrix blank
11. Second set of calibration standards
12. 2–3 Matrix blanks

Analyte absolute recovery and matrix effect

Analyte absolute recoveries were studied by comparing the analytes' instrument responses (peak areas) between prespiked and post spiked QC samples at low (1 ng/mL in whole blood) and high (10 ng/mL in whole blood) levels. Prespiked QCs were spiked appropriately in whole blood directly, and samples were prepared with the developed method. Post spiked QCs were spiked during the sample reconstitution step using the appropriate neat standard solution. Matrix effects were studied by comparing the analytes' instrument responses (peak areas) between post spiked QC samples and corresponding neat standards made in reagent blank. Matrix cleanup was investigated by monitoring the phospholipids profile.

Sample extraction

Figure 2 describes the sample preparation procedure. Before the sample preparation, a 96-well collection plate was placed under the Captiva EMR—Lipid plate. The stack went through the steps until the eluent was collected. Whole blood was protein precipitated in-well by first adding the blood sample, followed by crashing solvent. This addition order improved sample mixing homogeneity significantly, and ensured complete protein precipitation. The depth filtration design of the Captiva EMR—Lipid plate improved smooth sample elution without clogging. This study tested and verified two sample elution methods by PPM and centrifugation, respectively.

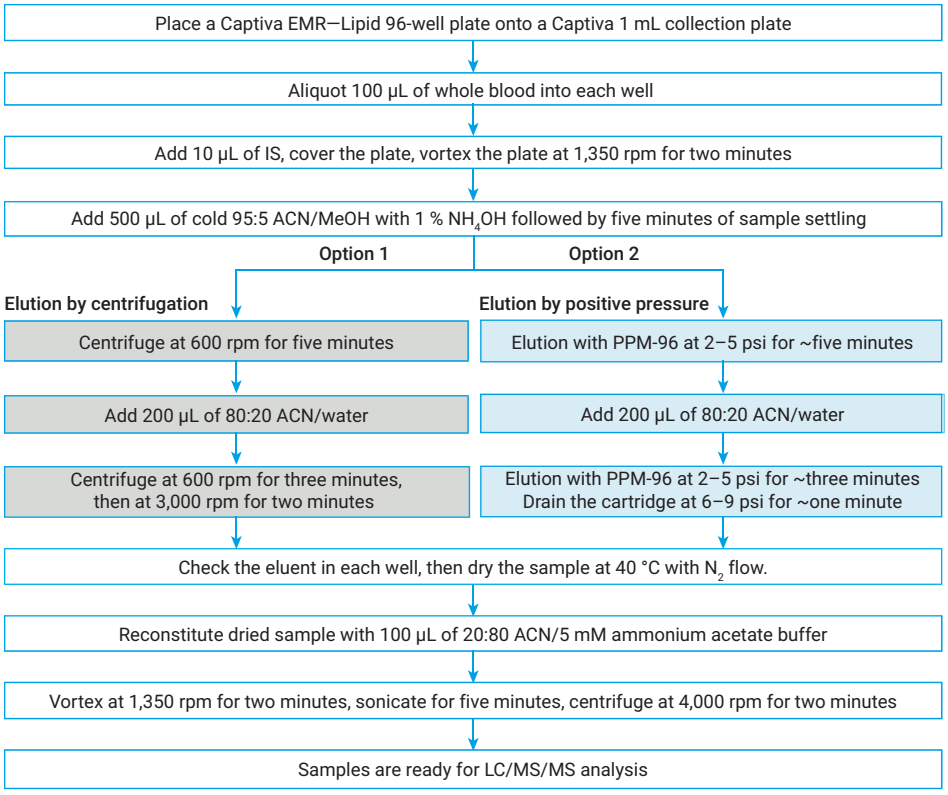


Figure 2. Human whole blood samples preparation procedure using in-well protein precipitation followed by Captiva EMR—Lipid cleanup. Samples were prepared as a batch in 96-well plate format.

Results and discussion

Method development

Whole blood in-well PPT: Human whole blood is considered a highly viscous body fluid, therefore whole blood sample handling and preparation are usually challenging. Unlike plasma and serum, whole blood contains blood cells and more proteins, which generate more precipitates during PPT. It is recommended that whole blood is added first, followed by crashing solvent for in-well PPT on 96-well EMR—Lipid plates. This addition order is essential to provide better PPT efficiency, simplify the workflow, and reduce the risk of sample loss and cross-contamination[9].

Crashing solvent: It has been reported that the use of cold MeOH/ACN solvent is a convenient approach to rupture red blood cells, release their contents (cytoplasm) into the surrounding blood plasma, and thus form powdery precipitates[10]. The 15:85 MeOH/ACN was used initially based on a previous study, but some analytes showed slightly lower recoveries. The crashing solvent was modified to 5:95 MeOH/ACN.

Since it is recommended to avoid using acidic crashing solvent for whole blood PPT[9], both neutral and basic crashing solvent were tested in this study. The PPT supernatant using neutral crashing solvent is slightly cloudy, indicating less effective PPT. The addition of NH_4OH (1 %) in crashing solvent improved method reproducibility with lower RSDs, and some analyte recovery slightly (Figure 3). As a result, 5:95 MeOH/ACN with 1 % NH_4OH was used as crashing solvent for whole blood in-well PPT.

Sample elution on EMR—Lipid plate: The Captiva EMR—Lipid plate format provides convenient pass-through cleanup for biological fluid extract after PPT, giving over 99 % of phospholipids removal[7]. To achieve satisfactory lipid removal efficiency, the appropriate flow rate during sample elution is important, as it provides better interaction between the sample and the EMR—Lipid sorbent. An elution flow rate of 1 drop per 3 to 5 seconds is highly recommended. For 96-well plate sample elution, there are three ways for elution control:

- Vacuum from bottom of plate
- Positive pressure from top of plate
- Centrifugation

Vacuum from bottom of plate was demonstrated in previous Application Notes[7,8], in which a 2–4" Hg vacuum was usually used for sample elution.

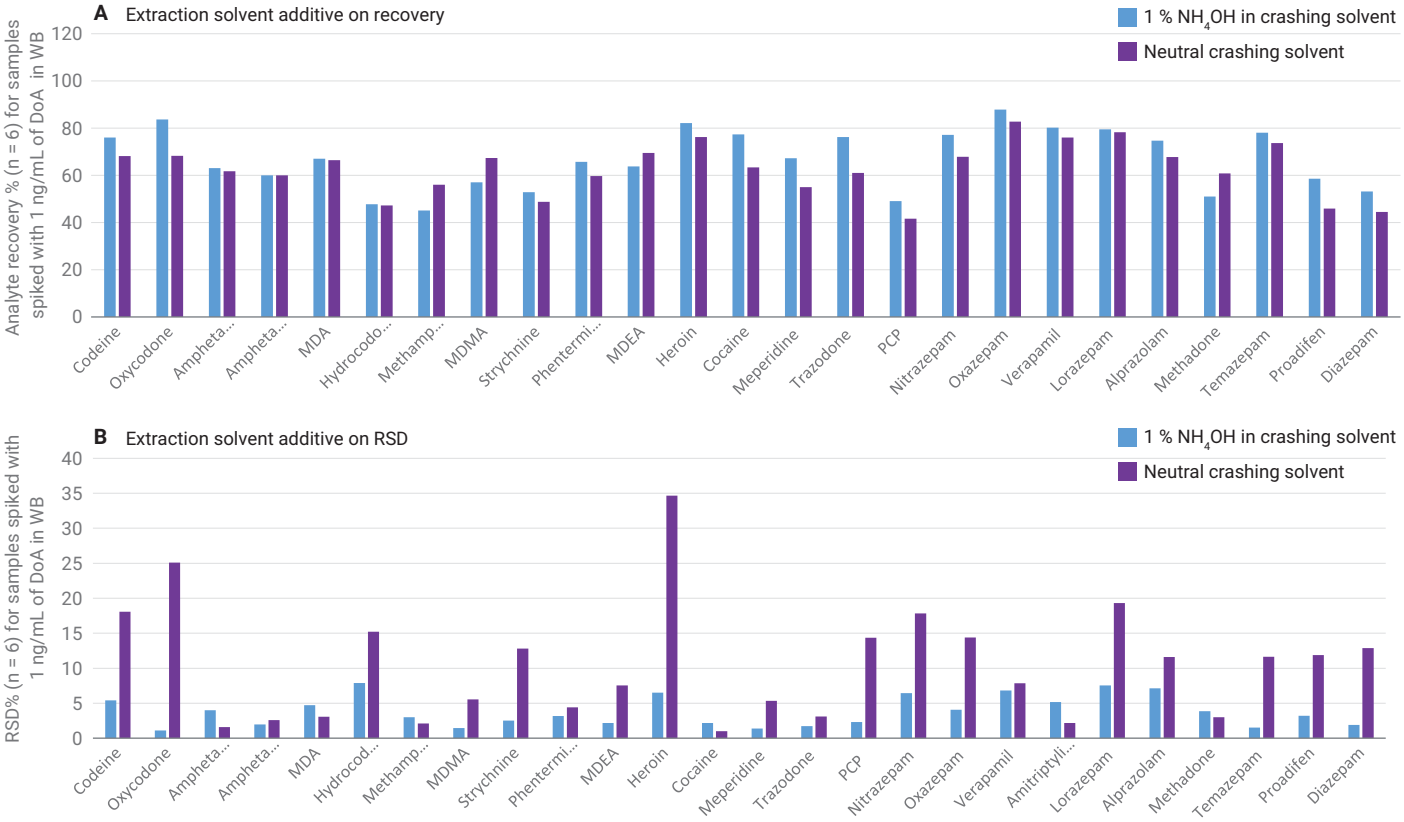


Figure 3. Comparison of crashing solvent additive (1 % NH_4OH) addition impact on analyte recovery and method reproducibility.

This study tested and verified elution by both positive pressure and centrifugation (Figure 2). For elution by PPM, a short centrifugation (30 seconds to 1 minute at 3,000 rpm) for the EMR—Lipid plate and collection plate stack after elution could be used to completely pull down all of eluent residue on the plate well walls. Both methods provided slow and consistent sample elution. Figure 4 shows the equivalent quantitative results obtained by both elution methods. Secondary elution was used by adding 200 µL of 80:20 ACN/water to the EMR—Lipid plate after the primary sample elution, improving analyte recovery by 10 to 20 % overall.

Method verification

The optimized method was then verified by three-day A&P runs to collect the complete quantitative results. The results shown in Table 4 include calibration curve data, limit of quantitation (LOQ), average recovery and matrix effect, accuracy and precision data for intra- and inter-day runs. Quantitation results for three A&P runs demonstrated excellent method accuracy and precision with all the intra-day and inter-day results meeting the acceptance criteria, defined as accuracy of 100 ±20 %, and RSD ≤20 %. An LOQ of 0.1 ng/mL in whole blood was established for most analytes, except 0.5 ng/mL for amphetamine, heroin, and lorazepam, due to either compound low sensitivity or matrix interferences. Linear regression and weight of 1/x were used for all of analyte calibration curves with a correlation coefficient R^2 >0.99. Most DoA analytes gave >70 % absolute recoveries, with a few exceptions in the sixties. The method provided superior precision for all analytes at different spiking concentrations across the calibration range, and sufficient sensitivity to meet the desired LOQ. The developed method was verified across three-day runs using centrifuge elution, then cross-verified by a one-day run using positive pressure elution.

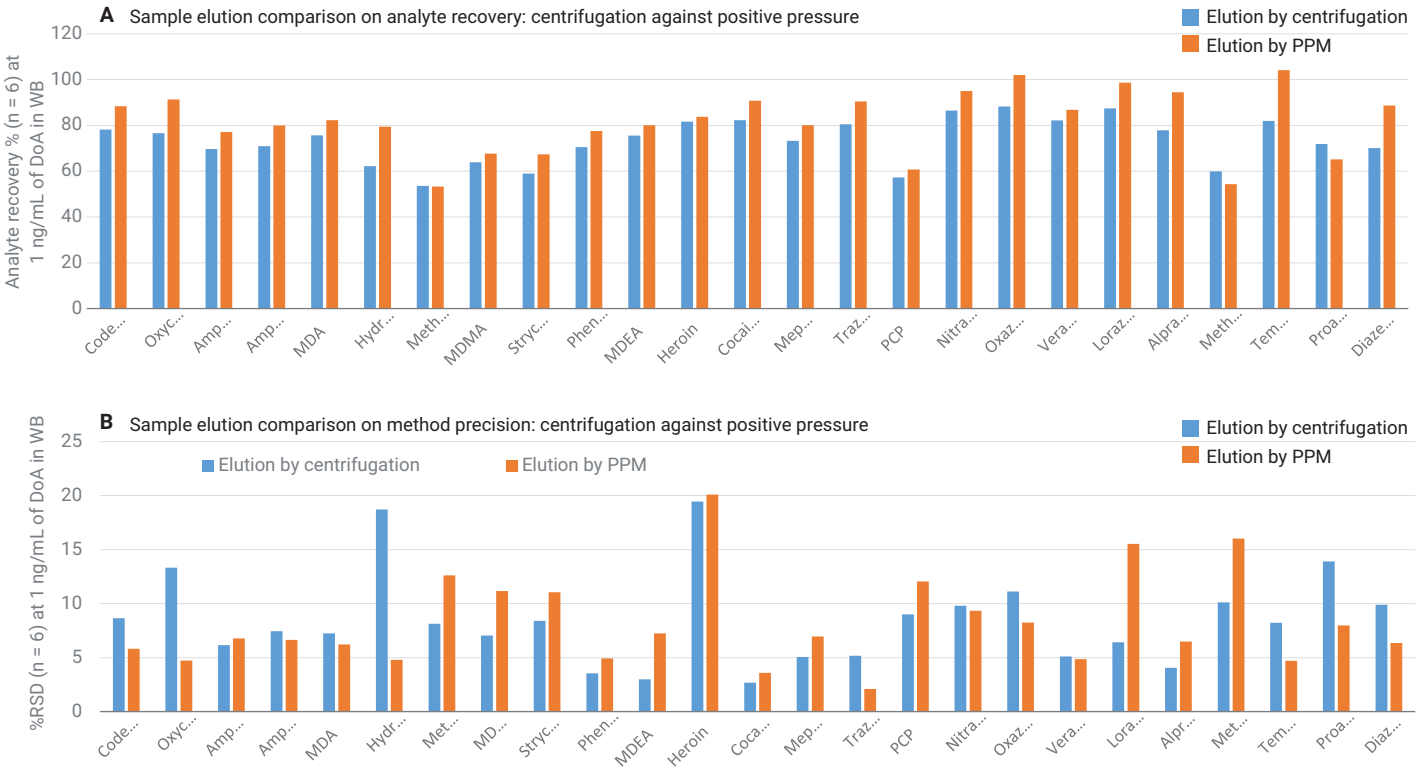


Figure 4. Analyte recovery (A) and method precision (B) comparison for two different elution methods: by centrifugation and positive pressure.

Matrix cleanup and impact on instrument detection system

Matrix cleanup was assessed by monitoring the phospholipids profile using the precursor ion scan for 184 product ion. When compared to samples with PPT only, >99 % of phospholipids were removed by EMR—Lipid cleanup (Figure 5). This result corresponds well with previous results[7]. The removal of phospholipids not only improved the method reliability and quantitation result consistency, but also significantly reduced the system contamination and carryover.

PPT alone has been widely used to prepare biological fluid samples for LC/MS/MS analysis. In addition to impacting method reliability and data quality, matrix interferences can also accumulate in the detection flowpath, such as injection port, LC column, MS source, and so on. Eventually, sample analysis can fail and result in more instrument downtime. Traditionally, the contamination and accumulation of matrix interferences on the detection flowpath can be reduced by implementing a longer LC gradient, more needle washes, or more blank sample injections after samples, or periodically system flushing use high organic mobile phase such as 100 % ACN. While these strategies may reduce the effect of matrix contamination/accumulation on instrument system, they are time-consuming and limit testing throughput.

A previous study[9] showed that running biological samples with EMR—Lipid cleanup can not only reduce detection system contamination and instrument downtime for cleaning, but also shorten the sample testing cycle time using a shorter LC gradient and less system washing time. These benefits significantly improve sample testing throughput and overall lab productivity.

Table 4. Method verification results for quantitative determination of 24 DoA compounds in human whole blood.

Analyte	LOQ (ng/mL)	Calibration range (ng/mL)	Correlation coefficient R^2	Average recovery % (n = 12)	Average ME% (n = 12)	Spiking concentration (ng/mL)	Results by centrifugation elution			Results by positive pressure elution	
							Accuracy% (n = 18)	Inter-day RSD% (n = 6)	Intra-day RSD% (n = 18)	Accuracy % (n = 6)	RSD% (n = 6)
Codeine	0.1	0.1–20	0.9909	87	13	0.1	97	9.3	6.8	97	11.0
						1	103	5.9	5.7	100	7.2
						20	112	3.0	3.0	100	4.9
Oxycodone	0.1	0.1–20	0.9952	81	25	0.1	102	6.0	5.8	95	5.2
						1	99	4.4	9.2	98	7.6
						20	108	2.4	2.9	105	3.8
Amphetamine	0.5	0.5–20	0.9947	79	-9	0.5	108	7.8	5.5	97	5.2
						1	101	2.5	8.1	97	3.4
						20	108	5.7	2.3	99	2.9
MDA	0.1	0.1–20	0.9973	84	4	0.1	102	6.9	6.4	100	7.0
						1	104	4.2	3.0	97	5.4
						20	108	5.8	4.3	99	4.7
Hydrocodone	0.1	0.1–20	0.9920	71	-7	0.1	102	7.8	8.4	101	8.8
						1	104	9.2	9.7	96	9.1
						20	109	8.3	5.8	107	5.6
Methamphetamine	0.1	0.1–20	0.9938	60	2	0.1	100	5.2	7.1	96	4.0
						1	103	10.4	9.5	96	3.7
						20	109	7.4	4.4	102	5.0
MDMA	0.1	0.1–20	0.9962	70	6	0.1	103	3.0	3.6	96	2.8
						1	103	5.8	6.5	97	2.9
						20	110	7.3	3.8	100	5.0
Strychnine	0.1	0.1–20	0.9912	66	5	0.1	103	4.7	5.6	92	6.6
						1	104	4.6	5.2	96	4.8
						20	111	6.7	4.8	105	6.6
Phentermine	0.1	0.1–20	0.9928	80	0	0.1	105	6.9	5.6	94	6.1
						1	106	6.9	4.9	95	3.2
						20	104	5.4	5.5	99	2.9
MDEA	0.1	0.1–20	0.9971	79	7	0.1	102	4.0	4.2	101	5.6
						1	106	5.2	4.2	93	4.5
						20	109	5.7	3.9	98	4.0
Heroin	0.5	0.5–20	0.9943	80	25	0.1	97	13.4	16.6	92	11.5
						1	100	6.8	7.0	102	10.7
						20	107	6.6	8.5	94	8.2
Cocaine	0.1	0.1–20	0.9987	88	-19	0.1	106	4.0	5.8	105	12.5
						1	104	4.7	4.2	96	4.3
						20	109	2.2	3.4	103	3.1
Meperidine	0.1	0.1–20	0.9948	79	-14	0.1	109	3.9	4.7	100	2.9
						1	107	4.7	2.7	94	1.9
						20	109	4.8	3.0	105	5.9
Trazodone	0.1	0.1–20	0.9957	87	-7	0.1	110	5.6	3.7	104	4.5
						1	105	3.8	3.6	96	4.4
						20	111	4.8	3.6	107	4.5
PCP	0.1	0.1–20	0.9916	68	-26	0.1	104	6.9	7.2	102	2.9
						1	104	9.8	9.2	99	2.0
						20	106	9.9	7.8	102	4.0
Nitrazepam	0.1	0.1–20	0.9941	91	7	0.1	100	11.2	9.3	116	12.5
						1	106	6.7	7.6	105	6.2
						20	109	5.5	5.8	100	6.3

Table 4. Method verification results for quantitative determination of 24 DoA compounds in human whole blood (continued).

Analyte	LOQ (ng/mL)	Calibration range (ng/mL)	Correlation coefficient R^2	Average recovery % (n = 12)	Average ME% (n = 12)	Spiking concentration (ng/mL)	Results by centrifugation elution			Results by positive pressure elution	
							Accuracy% (n = 18)	Inter-day RSD% (n = 6)	Intra-day RSD% (n = 18)	Accuracy % (n = 6)	RSD% (n = 6)
Oxazepam	0.1	0.1–20	0.9953	88	32	0.1	102	7.4	13.7	93	12.7
						1	103	7.0	9.0	101	11.9
						20	114	5.5	5.9	108	6.3
Verapamil	0.1	0.1–20	0.9959	79	31	0.1	111	2.7	3.1	100	1.6
						1	100	5.6	4.6	92	5.7
						20	112	3.1	7.0	103	7.9
Lorazepam	0.5	0.5–20	0.9947	88	20	0.1	99	11.3	13.6	102	10.9
						1	100	6.3	10.1	103	9.8
						20	109	1.9	3.7	105	4.9
Alprazolam	0.1	0.1–20	0.9949	86	19	0.1	100	5.5	7.6	101	6.8
						1	107	4.7	4.2	97	7.2
						20	104	3.8	3.8	102	5.3
Methadone	0.1	0.1–20	0.9950	62	5	0.1	102	4.4	8.6	99	4.8
						1	101	7.7	8.2	93	3.0
						20	110	6.5	4.8	103	6.1
Temazepam	0.1	0.1–20	0.9947	85	17	0.1	105	12.1	12.2	106	16.7
						1	107	6.0	5.5	94	13.6
						20	111	1.3	4.0	104	3.2
Proadifen	0.1	0.1–20	0.9945	70	5	0.1	109	3.0	4.0	102	7.0
						1	96	3.6	3.0	92	8.9
						20	112	3.5	5.6	110	5.7
Diazepam	0.1	0.1–20	0.9977	72	2	0.1	108	3.8	6.8	88	9.0
						1	106	5.5	4.1	94	3.9
						20	110	2.6	3.5	101	7.7

Conclusions

A sample preparation method using protein precipitation followed by Captiva EMR—Lipid cleanup was verified for quantitative determination of 24 representative DoA compounds in human whole blood. Three-day accuracy and precision runs verified that this method provides excellent and tight calibration curve linearity, exceptional intra- and inter-day accuracy and precision, and acceptable analyte recovery and matrix effects. Captiva EMR—Lipid cleanup provided excellent phospholipid removal from the whole blood matrix. In-well PPT was accomplished by adding the whole blood sample first, then the crashing solvent to improve sample mixing homogeneity and encourage complete precipitation of proteins. The developed protocol on

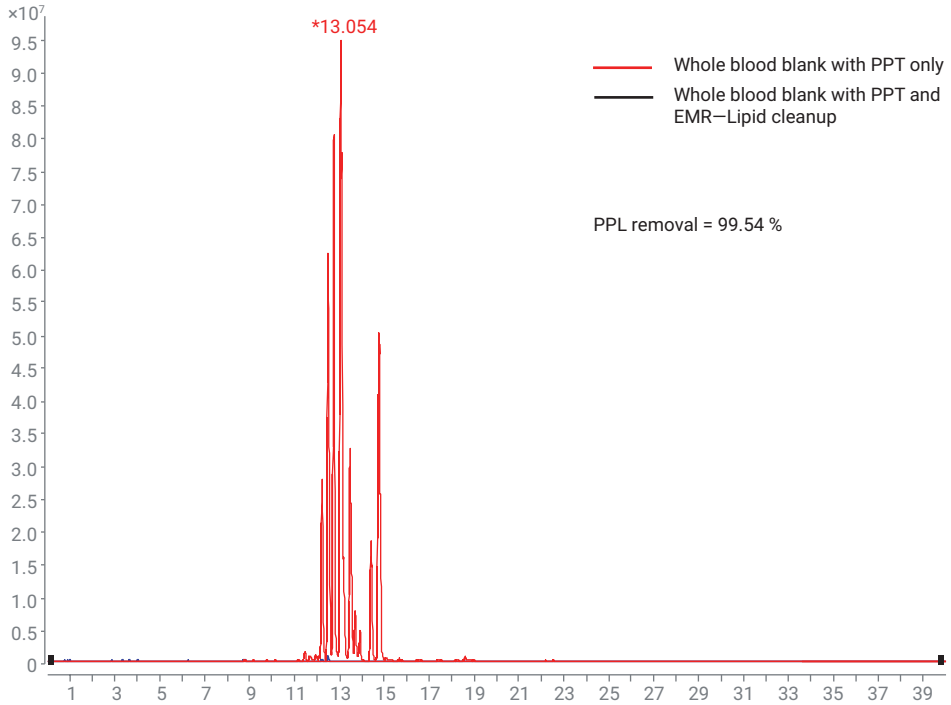


Figure 5. Overplayed chromatograms for phospholipids profile by monitoring a precursor ion scan for 184 *m/z* between whole blood sample prepared by PPT without and with Captiva EMR—Lipid cleanup.

96-well plate format is suitable for fast and automatable sample preparation needs in high throughput labs, the convenient in-well PPT followed with EMR—Lipid cleanup simplifies the workflow while still providing efficient sample extraction and matrix cleanup. Cleaner samples for analysis also reduce the time needed to clean the instrumental detection system, which improves sample testing throughput and data quality.

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