Application Note

Clinical Research

Drug Analysis in Human Urine Using Agilent Chem Elut S Supported Liquid Extraction by LC/MS/MS

Author

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Abstract

Agilent Chem Elut S is a new sample preparation product that uses synthetic sorbent for supported liquid extraction (SLE). This application note demonstrates the use of Chem Elut S 96-well plates (400 mg) for the quantitative analysis of 24 drugs in human urine by LC/MS/MS. Urine samples were enzyme-digested with β-glucuronidase then loaded into Chem Elut S 400 mg 96-well plates. Aqueous sample was absorbed onto the media and equilibrated for five minutes, then eluted with methyl *tert*-butyl ether (MTBE) to give a clean extract containing target analytes. The method was verified for accuracy and precision, and delivered excellent recovery (100 ±20%) and precision (%RSD <20%) for all but one compound. Significant advantages are demonstrated against traditional liquid-liquid extraction (LLE) and diatomaceous earth SLE methods. Results indicate that Chem Elut S, based on synthetic SLE sorbent, provides fast, simple, and consistent sample preparation for this application.

Introduction

In clinical research, fast and reliable drug quantitation in biological specimens is of great importance for improving methods to analyze emerging drugs of interest to ultimately improve patient care. To achieve their analytical goals, clinical researchers need sample preparation techniques that are high quality to deliver the best possible results, with maximum efficiency to ensure the best possible turnaround times. Urine matrix is not as complex as other biological matrices, such as plasma and serum. However, it contains a large amount of various salts, which can interfere with the test's reliability and instrument usability. Therefore, appropriate sample preparation is necessary for matrix cleanup and drug extraction. Additionally, drug analytes are often glycosylated and require enzymatic digestion with β-glucuronidase prior to sample preparation to release the free drugs for the following extraction and analysis.[4]

Common techniques for preparing samples for drug analysis include LLE, solid phase extraction (SPE), and SLE. LLE can be particularly labor-intensive, requiring extensive sample mixing, manual phase separation, and occasionally centrifugation to break up emulsions. Furthermore, LLE is difficult and cumbersome to automate, in contrast to SLE. In traditional SLE, an aqueous sample is loaded onto the sorbent bed containing diatomaceous earth (DE), where the aqueous sample is coated as a thin film on the material. A water-immiscible solvent is then passed through the SLE bed, extracting target analytes from the sample and eluting them into a collection tube for post-treatment and analysis. Compared to traditional LLE, the interaction surface between aqueous and organic phases using SLE is significantly increased, which improves analyte partition from the aqueous to organic phase and obsoletes the mixing step. This provides significant time and labor savings over conventional LLE with simplified and improved reproducibility.

Additionally, DE is a natural material, consisting of irregular fossilized microorganisms, and it is difficult to control the sorbent batch-to-batch particle consistency. The sorbent variability complicates product manufacturing and quality control, and leads to product performance inconsistency. DE can give lower and inconsistent water-holding capacity when compared to synthetic media. The Chem Elut S sorbent greatly improves water-holding capacity, batch-to-batch consistency, and performance consistency. The 96-well plate design offers large headspace for samples and eluent, a square upper frit that holds sample until pressure/vacuum is applied, a full skirt for hardware compatibility, and fast, consistent elution.

This study uses Chem Elut S 2 mL 96-well plates (400 µL) to quantitatively determine 24 drugs in human urine. Experimental comparisons were also evaluated between the Chem Elut S, LLE, and DE-based SLE based on recovery, reproducibility, and ease-of-use.

Experimental

All reagents and solvents were HPLC or analytical grade. Methanol (MeOH) and acetonitrile (ACN) was from Honeywell (Muskegon, MI, USA), and methyl *tert*-butyl ether (MTBE) was from VWR-BDH Chemicals (Radnor, PA, USA). A concentrated solution of hydrochloric acid (HCl, 38%) was obtained from VWR. A combined drug standard stock solution was from Agilent Technologies (part number 5190-0470), and internal standard (IS) stock solutions (1 mg/mL) were from Cerilliant (Round Rock, TX, USA). β-Glucuronidase enzyme was purchased as a solution (100,000 units/mL) from Sigma-Aldrich (St. Louis, MO, USA). Human urine (Mass Spect Gold) was purchased from Golden West Biologicals, Inc. (Temecula, CA, USA). Reagent solutions were prepared fresh for every batch to avoid variability.

Standards and solutions

Combined standard and IS intermediate spiking solutions were prepared at 20 µg/mL in MeOH with the dilution of appropriate volume of individual stock solutions. All spiking solutions are stored at –20 °C until use.

Equipment and supplies

- Agilent Chem Elut S 400 µL (part number 5610-2004)
- Agilent positive pressure manifold, PPM-96 (part number 5191-4116)
- Agilent square 96-well 2 mL collection plate (part number 5133009)
- Agilent square 96-well sealing caps (part number 5133005)
- SPE 96 evaporator
- Multitube Vortexer (VWR, PA, USA)
- Eppendorf pipettes and repeater
- ViaFlo 96 liquid handler (Integra, Hudson, NH, USA)

Instrument conditions

- Agilent 1290 Infinity quaternary pump (G4204A)
- Agilent 1290 Infinity autosampler (G4226A)
- Agilent 1290 Infinity thermostatted column compartment (G1316C)
- Agilent 1290 Infinity thermostat (G1330B)
- Agilent 6490 triple quadrupole LC/MS with iFunnel

Table 1 shows the analyte acquisition parameters, and Figure 1 shows the LC/MS/MS chromatogram of 1 ng/mL drugs in urine. See Table 1 for peak identification following the elution order.

LC Parameters	
Analytical Column	Agilent Poroshell 120 EC-C8, 2.7 µm, 2.1 × 100 mm (695775-906T) Agilent Poroshell 120 EC-C18, 2.1 × 5 mm, 2.7 µm, guard column (821725-911)
Column Temperature	50 °C
Injection Volume	2 µL
Mobile Phase A	5 mM ammonium formate + 0.1% formic acid in water
Mobile Phase B	0.1% formic acid in ACN
Flow Rate	0.5 mL/min
Gradient	Hold at 5% B to 0.5 minutes, Ramp to 50% B at 5 minutes, Ramp to 95% B at 6 minutes, Hold at 95% B to 7 minutes
Post Time	2 minutes

MS/MS configuration and parameters

Agilent 6490 Triple Quadrupole LC/MS with iFunnel (G6490A)	
MS/MS Mode	Dynamic MRM
Ion mode	Positive
Drying Gas Temperature	250 °C
Drying Gas Flow	5 L/min
Nebulizer Pressure	45 psi
Sheath Gas Temperature	325 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	3,000 V
EMV	200 V
Nozzle Voltage	1,500 V
iFunnel Parameters	High-pressure RF: 90(Pos), 90(Neg) Low-pressure RF: 70(Pos), 60(Neg)

Table 1. Target drugs, retention time, and MRM parameters.

Analyte	Internal Standard	Retention Time (min)	Precursor Ion (*m/z*)	Product Ion (*m/z*)			
				Quantifier Ion	CE (V)	Qualifier Ion	CE (V)
Codeine	IS 1	2.35	300.2	128.1	60	165.1	40
Oxycodone	IS 1	2.68	316.2	241.1	28	256.1	24
Amphetamine	IS 1	2.64	136.1	93.0	13	124.1	5
Amphetamine-d_3 (IS 1)		2.64	141.1	91.1	20	119.1	10
MDA	IS 1	2.72	180.1	163.1	4	105.1	24
Hydrocodone	IS 1	2.84	300.2	128.1	60	199.1	28
Methamphetamine	IS 1	2.87	150.1	91.1	20	119.1	8
MDMA	IS 1	2.91	194.1	163.1	8	105.1	24
Strychnine	IS 1	3.10	335.2	184.1	40	156.1	40
Phentermine	IS 1	3.10	150.1	133.1	8	133.1	8
MDEA	IS 1	3.19	208.1	163.1	8	105.1	48
Heroin	IS 2	3.81	370.2	328.2	20	165.1	24
Cocaine	IS 2	3.94	304.2	182.1	16	82.0	40
Cocaine-d_3 (IS 2)		3.94	307.2	185.1	30	82.0	48
Meperidine	IS 2	4.03	248.2	220.1	20	174.1	48
Trazodone	IS 2	4.35	372.2	176.1	24	148.1	16
PCP	IS 2	4.51	244.2	159.1	8	159.1	8
Nitrazepam	IS 2	5.20	282.1	180.1	40	236.1	24
Oxazepam	IS 2	5.27	287.1	241.1	20	104.1	40
Verapamil	IS 2	5.44	455.3	165.1	28	150.1	48
Lorazepam	IS 3	5.41	321.0	229.1	32	275.0	20
Alprazolam	IS 3	5.51	309.1	205.1	48	281.1	40
Methadone	IS 3	5.54	310.2	265.2	12	105.0	28
Temazepam	IS 3	5.73	301.1	177.0	44	255.1	16
Proadifen	IS 3	6.24	354.2	167.1	40	209.1	20
Diazepam	IS 3	6.18	285.1	193.1	32	154.1	24
Diazepam-d_3 (IS 3)		6.18	290.1	198.1	32	154.1	24

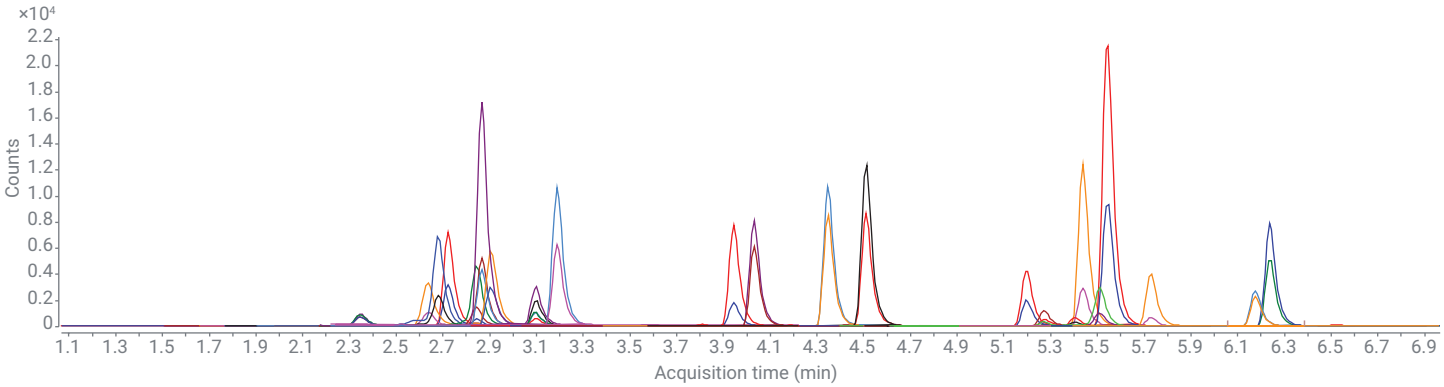

Figure 1. LC/MS/MS MRM chromatogram overlay of 24 drugs at 1 ng/mL in urine after Agilent Chem Elut S treatment.

Calibration standards and QC sample preparation

The drug standard spiking solution (1 µg/mL) was used to prepare calibration curve standards in human urine. The dynamic range for the calibration curve was 0.1 to 20 ng/mL, including 0.1, 0.5, 1, 5, 10, 15, and 20 ng/mL standards. Neat calibration standard solutions were prepared in 85:15 mobile phase A:ACN, then used to reconstitute matrix blanks to generate matrix-matched calibration curves. Three levels of quality control (QC) samples were spiked in urine prior to extraction, and extracted and run for method recovery and precision verification, including 0.1 ng/mL limit of quantitation (LOQ), except 0.5 ng/mL for amphetamine and heroin, 1 ng/mL (mid-QC), and 20 ng/mL (high-QC). Internal standards were spiked into QCs, calibrators, and blanks at 200 ng/mL.

Sample preparation procedure

The sample preparation procedure was based on the SLE general protocol shown in Figure 2. The four steps include:

1. Load the aqueous sample with gentle positive pressure/vacuum.
2. Equilibrate the sample in Chem Elut S sorbent for five minutes.
3. Add a water immiscible solvent/solvent mixture to extract the analytes.
4. Elute the organic solvent with gravity, then applying positive pressure/vacuum to drain the plate.

Urine samples benefit from pretreatment hydrolysis with β-glucuronidase to deglycosylate drugs prior to sample extraction. Several water-immiscible solvents were screened, including MTBE, ethyl acetate (EA), and dichloromethane (DCM); MTBE was selected due to higher recovery and reproducibility.

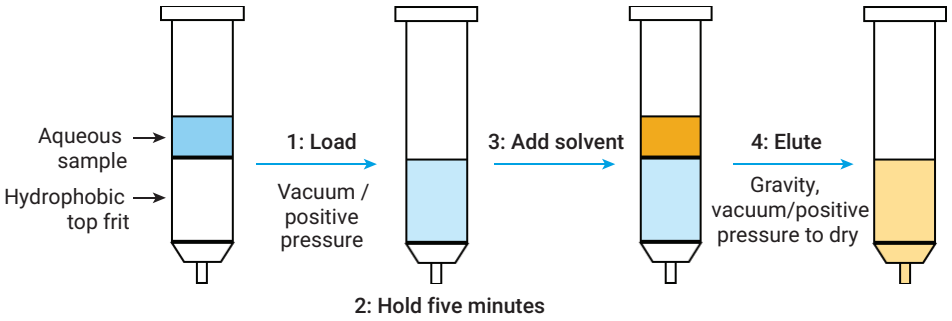

Figure 2. Diagram of general Agilent Chem Elut S workflow.

Enzyme hydrolysis

1. Aliquot 200 µL urine samples into a 2 mL collection plate, add 175 µL of 100 mM ammonium acetate (pH 4) and 25 µL β-glucuronidase solution (100,000 units/mL).
2. Mix and vortex for 30 seconds and incubate at 40 °C (water bath) for 60 minutes.
3. Bring samples to room temperature, add 20 µL of 5 M ammonium hydroxide, and vortex to quench the enzyme reaction.

SLE extraction procedure

1. Use a 96-probe liquid handler to transfer entire samples from the collection plate into the Chem Elut S 400 µL plate with another 2 mL collection plate beneath. Apply 2 to 3 psi pressure to drive the aqueous sample into the sorbent until no visible liquid is left in the wells.
2. Equilibrate for five minutes.
3. Add 900 µL of organic solvent into the wells, and allow the solvent to elute by gravity. Repeat with a second 900 µL elution volume of organic solvent (1,800 µL total).
4. Apply 3 to 5 psi pressure for 20 to 30 seconds to completely dry the SLE bed.

Evaporation and reconstitution

1. Add 10 µL of 10% HCl solution to each well. This step is optional, and only applied with unacceptable reproducibility observed for volatile drugs such as amphetamine.
2. Place collection plate in a 96-well evaporator, and evaporate to dryness at 40 °C under nitrogen.
3. Reconstitute with 200 µL reconstitution solution (85:15 mobile phase A:ACN) or neat calibration standard solutions as appropriate.
4. Add top plate mat, vortex, sonicate, centrifuge, and place in autosampler for LC/MS/MS analysis.

Method verification

The Chem Elut S method for drug analysis in urine was tested for recovery and reproducibility. Batches consisted of 2x double blanks, 8x blanks, two sets of 7x calibrators, and 6x QC at each level, where QC samples were bracketed between two sets of calibration curves. Isotopic internal standards amphetamine-d_3, cocaine-d_3, and diazapam-d_5 were spiked at 200 ng/mL in urine before extraction.

Results and discussion

Linearity

The data were processed with Agilent Mass Hunter Quantitative Analysis software. Calibration curves gave R^2 values between 0.991 and 0.999 for all target drugs using linear regression fitting and $1/x^2$ weighting.

Recovery and reproducibility

The study produced good results, as shown by the summary in Table 2. Recoveries for all but one analyte, proadifen, were 79.3 to 117.4% and %RSD <16. Proadifen consistently gave low recovery (approximately 55% for both SLE and LLE methods, but good sensitivity and high reproducibility (%RSD 6.2 to 11.2). The 0.1 ng/mL LOQ was achievable for all compounds except amphetamine and heroin (LOQ = 0.5 ng/mL), due to either matrix interference or method sensitivity.

Table 2. Experimental parameters and results for drugs of abuse in urine using an optimized Chem Elut S procedure.

Analyte	LOQ (ng/mL)	Calibration Range (ng/mL)	Correlation Coefficient (R^2)	Avg ME (% n = 6)	Spiking Concentration (ng/mL)	Average Recovery % (n = 6)	%RSD (n = 6)
Codeine	0.1	0.1 – 20	0.9973	0	0.1	84.8	12.5
					1	103.4	7.1
					20	93.3	9.7
Oxycodone	0.1	0.1 – 20	0.9915	–8	0.1	91.3	4.3
					1	95.3	2.7
					20	92.4	1.3
Amphetamine	0.5	0.5 – 20	0.9932	–3	0.5	103.1	4.0
					1	106.6	3.9
					20	98.9	5.6
MDA	0.1	0.1 – 20	0.9946	–7	0.1	89.6	5.5
					1	107.5	4.0
					20	96.5	4.8
Hydrocodone	0.1	0.1 – 20	0.9981	–14	0.1	112.1	4.0
					1	104.7	8.7
					20	98.3	3.6
Methamphetamine	0.1	0.1 – 20	0.9977	–4	0.1	102.2	7.0
					1	105.3	3.8
					20	103.3	3.9
MDMA	0.1	0.1 – 20	0.9979	–4	0.1	83.5	6.7
					1	89.2	2.7
					20	88.4	7.6
Strychnine	0.1	0.1 – 20	0.9966	–8	0.1	91.1	15.7
					1	90.8	4.7
					20	90.1	3.0
Phentermine	0.1	0.1 – 20	0.9942	–1	0.1	89.6	8.6
					1	112.8	3.9
					20	101.7	5.4
MDEA	0.1	0.1 – 20	0.9991	–11	0.1	98.8	4.4
					1	99.9	6.2
					20	98.9	5.2
Heroin	0.5	0.5 – 20	0.9909	–9	0.5	84.5	6.3
					1	79.3	7.6
					20	86.8	4.1
Cocaine	0.1	0.1 – 20	0.9925	–2	0.1	93.0	7.5
					1	98.5	10.3
					20	97.6	9.7

Analyte	LOQ (ng/mL)	Calibration Range (ng/mL)	Correlation Coefficient (R^2)	Avg ME (% n = 6)	Spiking Concentration (ng/mL)	Average Recovery % (n = 6)	%RSD (n = 6)
Meperidine	0.1	0.1 – 20	0.9982	–5	0.1	113.1	2.6
					1	117.2	8.9
					20	107.3	7.1
Trazodone	0.1	0.1 – 20	0.9981	–4	0.1	109.2	6.6
					1	109.9	11.4
					20	107.3	4.8
PCP	0.1	0.1 – 20	0.9944	–15	0.1	102.6	4.2
					1	101.2	11.8
					20	96.6	3.1
Nitrazepam	0.1	0.1 – 20	0.9957	9	0.1	99.7	6.9
					1	109.5	14.9
					20	107.9	9.1
Oxazepam	0.1	0.1 – 20	0.9914	–9	0.1	116.4	12.0
					1	105.0	15.6
					20	86.8	9.6
Verapamil	0.1	0.1 – 20	0.9968	–6	0.1	93.2	6.1
					1	86.7	9.7
					20	88.9	6.6
Lorazepam	0.1	0.1 – 20	0.9920	0	0.1	105.3	9.0
					1	106.8	8.5
					20	97.6	11.2
Alprazolam	0.1	0.1 – 20	0.9985	–7	0.1	117.2	9.6
					1	111.1	9.3
					20	105.8	5.1
Methadone	0.1	0.1 – 20	0.9957	–6	0.1	102.3	5.7
					1	98.3	8.9
					20	97.3	6.5
Temazepam	0.1	0.1 – 20	0.9976	–8	0.1	117.4	8.1
					1	112.4	10.9
					20	93.5	9.8
Proadifen	0.1	0.1 – 20	0.9983	0	0.1	57.9	6.9
					1	52.8	11.2
					20	59.1	6.2
Diazepam	0.1	0.1 – 20	0.9969	1	0.1	94.3	5.7
					1	118.7	10.9
					20	111.3	5.7

Method and product comparison

Chem Elut S was also compared with LLE and a competitor's DE-based SLE for analyte recoveries and reproducibility (Figure 3). Analyte recoveries were studied by comparing the analytes' peak area with prespiked and postspiked QC samples at 1 ng/mL in urine. Prespiked QCs were spiked appropriately in blank urine, and samples were prepared with the developed SLE method. Postspike QCs were prepared by reconstituting with 1 ng/mL standards in matrix blanks after extraction.

The same procedures were used for DE-based SLE and Chem Elut S. The LLE used the same pretreatment, sample, and solvent volumes, but required manual transfer of MTBE with a Pasteur pipette to separate phases. As a result, the LLE took >50% longer than SLE and required significantly more of the analyst's effort and time to detail.

As expected, Chem Elut S gave excellent recovery and precision for all drugs except proadifen. The competitor's DE-based SLE gave acceptable recoveries for most analytes, but with significantly lower recoveries for several compounds. The reproducibility was especially poor for DE-based SLE, with six compounds over 20% RSD for six replicates. The inconsistent DE sorbent aqueous-holding capacity resulted in variable sample breakthrough well-to-well, which caused sample inconsistencies during sample preparation. The LLE method gave recovery comparable to the SLE method except much lower for oxycodone and hydrocodone. Comparatively, Chem Elut S delivers superior recovery and precision compared to LLE and the competitor's DE-based SLE. The same results are also reflected in other biological matrices, such as plasma, and will be highlighted in separate applications.[1]

Sample preparation with Chem Elut S

The Chem Elut S plates were simple to use, fast, and gave high analyte recovery and precision for drugs. The synthetic media is carefully manufactured to provide high sample-holding capacity, uniform packing, and optimal flow characteristics. Chem Elut S plates are optimized for SLE to include clean plastics, large headspace volume, full skirt, and a square top frit capable of holding aqueous samples for 60 minutes or more until positive pressure/vacuum is applied. These features provide excellent data quality, ease-of-use, and efficient matrix removal (that is, salts and partial phospholipids), especially for complex biological samples such as plasma and serum.[1]

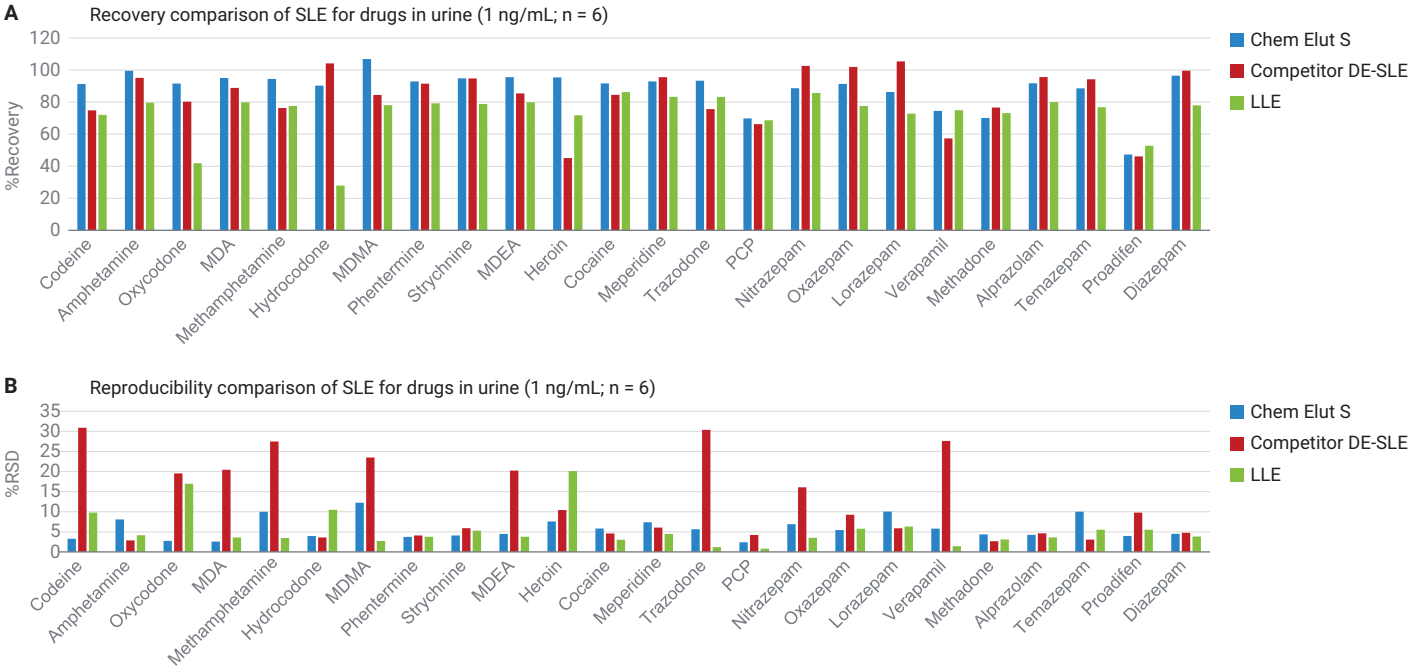

Figure 3. Comparison of recovery and reproducibility for drugs in urine for Agilent Chem Elut S, diatomaceous earth SLE, and LLE.

Conclusion

Agilent Chem Elut S uses a synthetic SLE media designed for consistency and high water-holding capacity, and delivers better recovery and reproducibility compared to DE-based SLE products. The developed method using Chem Elut S 400 µL plates delivered excellent recovery and reproducibility for drug analysis in urine, using a simplified platform for fast, consistent, high-throughput analysis, and quality results without the hassles associated with LLE. A wide variety of other sample and analyte types benefit from Chem Elut S, and will be featured in future publications.

Reference

1. Zhao, L. Quantitative Determination of a Panel of Endogenous Steroids in Human Serum by LC/MS/MS using Agilent Supported Liquid Extraction (SLE) Chem Elut S Plate. *Agilent Technologies Application Note*, publication number 5994-0949EN.

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